The Isolation of Bioactive Peptides from Collagen

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ABSTRACT

Wound healing involves a complex series of interactions between cells of the dermis and epidermis and their extracellular matrix (ECM). Studies suggest that the ECM, far from being an inactive bystander, actively orchestrates the key events in wound healing and regeneration. In particular, collagen and peptides derived from collagen have been shown to be chemotactic towards a variety of cell types including neutrophils, monocytes and fibroblasts. CNBr-derived collagen peptides also effect the attachment and migration of neural crest cells.

This study involved the isolation and purification of collagen peptides and characterisation of the effects of these peptides on cell behaviour. *In vitro* experiments were carried out on CNBr-derived peptides from collagens I and III, these were shown to stimulate fibroblast growth and chemotaxis. Peptides CB3 and CB8 from collagen α1(I) and peptides CB4 and CB8 from collagen α1(III) showed the most potent stimulation. A chemotactic peptide, 22 amino acids in length, obtained by sequential enzymatic digestion of collagen I with bacterial collagenase and chymotrypsin was isolated, this peptide was also found to be a potent chemoattractant for fibroblasts. A tetra-peptide with the amino acid sequence Gly-Pro-Ala-Gly was identified as one of the most chemotactically active regions in the collagen molecule.

The collagen peptide (22 amino acid sequence) was also tested *in vivo* for its ability to stimulate cellular infiltration and granulation tissue formation using the PVA (polyvinyl alcohol) model system in rats. The peptide was injected into PVA sponges which had been implanted into rats. After 7 or 10 days the sponges were removed and analysed both histologically and biochemically. The levels of collagen, DNA and protein in the PVA sponges injected with the peptide increased significantly compared to control sponges, after 10 days. This effect was concentration dependent, with the optimum tested concentration being 10μg/ml.

Experiments were also carried out on cell surface interactions. Binding of the peptide to fibroblasts was not inhibited by antibodies to integrins $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, suggesting that collagen-receptor interaction in this case may be non-integrin mediated.

From this work, it was shown that peptides isolated from collagens I and III were chemotactic for fibroblasts both *in vitro* and *in vivo.*

Declaration of originality

I declare that, unless otherwise stated, this thesis represents my own work and was composed by myself.

Christina Hamilton

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ABBREVIATIONS

BAEC	Bovine aorta endothelial cells
BM	Basement membrane
BSA	Bovine serum albumin
BSE	Bovine spongiform encephalitis
C5a	Complement factor C5a
$CaCl_2$	Calcium Chloride
cm	Centimetre(s)
CNBr	Cyanogen Bromide
CO_2	Carbon dioxide
DMEM	Dulbecco's modification of Eagle's medium
DM	Dermatomyositis
DNA	Deoxyribonucleic acid
EC	Endothelial cell
ECM	Extracellular matrix
EDTA	Ethylenediamine tetraacetic acid
EGF	Epidermal growth factor
FACIT	Fibril associated collagens with interrupted triple helix
FCS	Fetal calf serum
(b)FGF	(basic) Fibroblast growth factor
Fmoc/^{t}Bu	9-fluorenylmethoxycarbonyl tertiary butyl
FPLC	Fast protein liquid chromatography
g	Gram(s)
GAG	Glycosaminoglycan
GalNAc	N-acetlygalactosamine
GGBS	Glycylglycine buffered saline
GlcA	Glucuronic acid
GlcNAc	N-acetlyglucosamine
H_2O	Water
HA	Hyaluronic acid
HBSS	Hank's balanced salt solution
HCl	Hydrochloric acid
H&E	Haematoxylin and eosin
HPLC	High performance liquid chromatography
hr	Hour
IGF	Insulin-like growth factor
kDa	Kilodalton(s)
LDCF-F	Lymphocyte-derived chemotactic factor for fibroblasts
M	Molar
mA	Milliamp(s)
mg	Milligram(s)
ml	Millilitre(s)

mm	Millimetre(s)
mM	Millimolar
MMP	Matrix metalloproteinase(s)
MT	Matrixin
NaCl	Sodium chloride (salt)
NEM	N-ethylmaleimide
nm	Nanometre(s)
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
pg	Picogram(s)
PM	Polymyositis
PMN	Polymorphonuclear leukocyte(s)
PTH	Phenyl thiohydantoin
PVA	Polyvinyl alcohol
rpm	Revolutions per minute
RWF	Rat wound fibroblasts
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SMC	Smooth muscle cell(s)
TEMED	N,N,N',N'-tetramethylethylene diamine
TIMP	Tissue inhibitor of metalloproteinase (s)
TFA	Trifluoroacetic acid
TGF-α	Transforming growth factor alpha
TGF-β	Transforming growth factor beta
Tris	Tris(hydroxymethyl) amino methane
UV	Ultraviolet
µl	Microlitre(s)
µm	Micrometer(s)
v/v	Volume per volume
w/v	Weight per volume
w/w	Weight per weight
ZCl	Zyderm collagen implant

AMINO ACIDS

Three letter code	Amino Acid
Ala	Alanine
Cys	Cysteine
Asp	Aspartic
Glu	Glutamic acid
Phe	Phenylalanine
Gly	Glycine
His	Histidine
Ile	Isoleucine
Lys	Lysine
Leu	Leucine
Met	Methionine
Asn	Asparagine
Pro	Proline
Gln	Glutamine
Arg	Arginine
Ser	Serine
Thr	Threonine
Val	Valine
Trp	Tryptophan
Tyr	Tyrosine
Hyp	Hydroxyproline
Hyl	Hydroxylysine

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CHAPTER 1:

INTRODUCTION

1.1 WOUND HEALING

1.1.1 Introduction

Physical trauma has always faired high on the list of man's problems. Tissue repair is fundamental to guaranteeing the survival of living organisms. Wound treatment and healing is an ancient art (Majno 1975). Evidence of traumas with their related consequences have been found in vertebrate remains by archaeologists. Many skulls found from archaeological excavations have holes which show evidence of bone healing. Wounds can be dated back to our ancestor, *Australopitheus africanus,* who needed to deal with injuries as violence and war appeared in his culture (Majno 1975). Also by analogy with many modern races, ritual circumcision in both sexes was carried out so that the care of surgical wounds was by no means unknown to primitive man. In wound healing, the use of plants, biological fluids such as urine and blood, and animal products such as honey and pieces of fresh meat, mixed with folklore and magic, was common among ancient peoples (Doanati *et al.*, 1994). The first suturing of wounds by primitive man was by sewing them together with fibres or shreds of tendon. The oldest suture dates back to Ancient Egypt. The use of adhesives tapes and gum adhesives can be dated back four thousand years (Quinn 1996).

Wound healing is the complex response to tissue injury controlled by both local and migrated cells. Wound healing involves inflammation, proliferation of cells, e.g. fibroblasts, re-epithelisation, angiogenesis, synthesis and deposition of connective tissue and apoptosis, depending on the injury (reviews: Pierce & Mustoe 1995; Clark 1996; Martin 1997).

There is a complex series of events in the wound healing process between cells in the dermis and epidermis and their extracellular matrix (ECM). Many cell types, cytokines, coagulation factors, growth factors, complement activation and matrix

proteins such as fibronectin and collagen contribute to healing in various ways (Clark 1993). The functions and precise mechanisms of these cellular, humoral and local factors are unclear and poorly understood (Hunt & La Van 1989; Gailit & Clark 1994).

An injury may be defined as an interruption in the continuity of tissues. Repair of the injury is achieved primarily by proliferation, migration, and differentiation of involved cells (neutrophils, macrophages, fibroblasts, endothelial and epithelial cells). There are two processes involved in wound healing: repair and regeneration (Johnson 1990). The repair process is the replacement of tissue defects by a scar which binds the surrounding tissues together. All tissues and organs of the body, with the possible exception of teeth, are capable of repairing injuries (McMinn 1967, Needham 1952). Regeneration is the replacement of destroyed tissue with normal functioning cells of the type lost in the initial injury. Regeneration is under the control of positive and negative growth-regulating substances and is only possible in tissues that have a sustained population of cells capable of undergoing mitotic division (Lipowitz 1985). Neurons, for example are incapable of mitosis and hence regeneration.

Adult wound repair includes the stages of haemostasis, inflammation, proliferation and remodelling (Longaker & Adzick 1991). Haemostasis is the body's natural mechanism for preventing excessive bleeding, and involves vasoconstriction, platelet aggregation and degranulation, blood clotting and fibrin formation. Three major events occur during the inflammatory response; an increased blood supply, increased capillary permeability and cellular infiltration beginning with polymorphonuclear leukocytes (PMNs) and followed by macrophages and lymphocytes. This stage is responsible for defending against bacteria and for the secretion of numerous growth factors, cytokines and extracellular matrix components. The proliferative stage involves multiplication of fibroblasts, endothelial and epithelial cells. In the final remodelling stage there are progressive changes in the rates of synthesis of collagen types and crosslinks in order to form a mature scar (Quinn 1998).

1.1.2 Cellular Constituents of Wound Healing

Many cell types are actively involved in the wound healing process. Platelets adhere to exposed subendothelial collagen in damaged vessels where they release numerous vasoactive substances, growth factors which stimulate the proliferation of smooth muscle cells and fibroblasts (Clark 1993). Platelets thus play a role in the early initiation of fibroblast activity within the healing wound.

Neutrophils migrate into the wound within minutes of injury. Their role has been considered to be confined to defence against bacterial infection with their numbers decreasing in the absence of infection (Gabka *et al.*, 1995). However, studies have now shown that neutrophils are also a source of pro-inflammatory cytokines that serve as some of the earliest signals to activate local fibroblasts and keratinocytes (Martin 1997). In the absence of neutrophils there is increased infection and impaired wound healing, a lack of neutrophil-released factors is also detrimental (Harding & Leaper, 1998).

Macrophages, in contrast to neutrophils, have a central role. It is the crucial effector cell type that co-ordinates adult wound repair (Knighton & Fiegel 1989). Macrophages are phagocytic cells derived from monocytes and their cell numbers are maximal within 24 hours (Peacock 1984). These cells are important in the induction of fibroblast activity by the release of chemotactic substances (Hopkinson-Woolley *et al.*, 1994). Macrophages must be stimulated in order to induce this activity. Stimulated macrophages injected into the cornea induce fibroplasia, increased collagen synthesis, and neovascularisation (Robertson *et al.*, 1993). Macrophages also take part in wound debridement (i.e. removal of necrotic tissue and tissue remodelling). They are important producers of growth factors, which act as chemical signals triggering the activation and replication of cells, synthesis of structural proteins and vessel formation (Becker 1988). Transforming Growth Factor-beta

(TGF-β), Platelet Derived Growth Factor (PDGF), Fibroblast Growth Factor (FGF), Epidermal Growth Factor (EGF) and Transforming Growth Factor alpha (TGF-α) are common growth factors which are secreted at the site of the wound (Mian *et al.*, 1992).

Fibroblasts are responsible for production of collagens that fill the wound void. These cells migrate into the wound area by means of cytoplasmic extensions known as ruffled membranes, which use fibrin and fibronectin as a scaffold for migration (Probst & Bright 1985). Fibroblasts migrate into the wound before endothelial buds but behind macrophages. Further migration is inhibited by a process of contact inhibition when the ruffled membranes of two or more fibroblasts come together. Fibroblasts then begin secreting glycosaminoglycans and collagen which are responsible for binding the wound edges (Irvin 1981). In normal (uninjured) tissue, fibroblasts are sparsely distributed throughout the connective tissue matrix. After injury, fibroblasts are activated to migrate from adjacent tissue into the wound site, where they proliferate and produce collagen, elastin and proteoglycans, which then reconstruct the connective tissue. Fibroblasts can now be considered an important early participant in inflammatory responses (Hogaboam *et al.*, 1998). Fibroblasts can be activated (by bacterial products, in particular) to display new functions important in controlling extracellular matrix synthesis and producing cytokines. This signals the recruitment of immune cells to the site of infection (Smith *et al.*, 1997). It has recently been shown that in skin grafting the best healing was observed with a high number of fibroblasts cultured for 10 days prior to grafting (Lamme *et al.*, 2000).

Myofibroblasts are a unique group of smooth-muscle like fibroblasts that have a similar appearance and function regardless of residence (review: Powell *et al.*, 1999). These are specialised cells containing contractile filaments and were first described by Johnston in 1979. They play a central role in wound healing. The number present is proportional to the need for wound contraction. Myofibroblasts become activated and proliferate in the early stages of wounding. They respond to proinflammatory

cytokines and additional growth factors. As wound contraction and epithelialisation nears completion, the number of myofibroblasts decreases by apoptosis following repair or scar formation (Desmouliere *et al.*, 1995). Following migration into the wound, myofibroblasts form intercellular connections as well as attachments to the wound bed and surrounding dermis. Myofibroblasts contain smooth muscle myosin isoforms in addition to α-SM actin, which is responsible for the ability to contract and to move (Powell *et al.*, 1999). In mammals, Rho A, a small GTPase, acts on the actin cytoskeleton to cause change of shape or motility of myofibroblasts (Aspenstrom 1999). The contraction of the myofibroblast is responsible for wound contraction (Gabbiani 1992). Myofibroblasts express α and β integrins that are part of the adhesion mechanism of myofibroblasts to matrix proteins (Racine-Samson *et al.*, 1997).

T lymphocytes and their soluble mediators play a modulatory role in wound healing. Defined subsets of T cells express stimulatory or inhibitory effects on repair mechanisms and take part in a balanced system of wound cell regulation (Shaffer & Barbul 1998). B lymphocytes synthesise antibodies which provide immunity against invading organisms. These cells are also involved in the regulation of wound healing, mediated by release of cytokines/growth factors. Recent work has shown that human wound-associated lymphocyte populations are modulated during healing, with increased levels of both $CB8^+$ T-suppressor lymphocytes and B lymphocytes (Boyce *et al.*, 2000).

1.1.3 Stages of Wound Healing

It is convenient to describe the local process of wound healing in five stages although, in reality, this is a continuous process with one stage merging into the next. The timings are merely a guide and are relevant to both humans and animals. They can change depending on conditions i.e. temperature, infection, open wounds, chronic wounds.

1.1.3.1 Stage I: The haemostatic phase (seconds - minutes)

The first priority is to stop bleeding. The severed arterioles contract and the spilled blood clots. Blood is triggered to clot by either coming in contact with collagen or by mixing with a 'tissue factor' released by injured cells. Platelets also begin to pile up on the mouths of the bleeding vessels and create plugs called thrombi. The products of the clotting mechanisms help activate the healing process. Thrombin, the enzyme that generates fibrin, attracts and causes fibroblasts to replicate. Platelet-derived growth factor (PDGF), released by degranulating platelets, is also a mitogen and a chemotaxin for fibroblasts (review by Majno & Joris, 1996).

1.1.3.2 Stage II: The phase of traumatic inflammation (0-3 days)

This phase demonstrates all the features of acute inflammation due to infection (redness, swelling, heat), however infection plays no part. The phase begins within a few minutes of wounding and lasts for about three days. When tissue is disrupted, blood vessels are injured and bleed into the space created. Platelets and the coagulation system cause blood to clot around the wound. Injured blood vessels thrombose and the bleeding stops. Damaged tissue and mast cells secrete histamine and enzymes, causing vasodilation of surrounding capillaries and exudation of serum and white cells into the damaged area (Clark 1985). The increased blood supply with oedema and engorgement of surrounding vessels accounts for the inflammatory appearance, warmth and throbbing sensation experienced by the patient. At this stage, two important cell types arrive in the wound. Polymorphonuclear leukocytes and macrophages serve to combine in defence against bacteria and begin the process of repair by clearing debris, damaged tissue and blood clots (Turk *et al.*, 1966).

1.1.3.3 Stage III: The destructive phase (2-5 days)

Now the polymorphs and macrophages clear the wound of devitalised and unwanted material. Macrophages are essential to the repair process and play a central role whereas repair occurs even with a major reduction in the number of polymorphs. Macrophages attract further macrophage migration and stimulate the formation and multiplication of fibroblasts (Johnston 1990).

1.1.3.4 Stage IV: The proliferative (or fibroplasia) phase (3-24 days)

Fibroblasts line up behind the macrophages and begin to produce the fibres of collagen which are the main constituent of skin, tendons, ligaments, bones, cartilage, fascia and scar tissue. The peak rate of synthesis of new collagen in a primarily healing wound occurs around the 5th to the 7th day (Weber *et al.*, 1995). This considerable cellular and chemical activity during the proliferative phase results in the formation of 'granulation tissue'. Granulation tissue is a loose collection of fibroblasts, inflammatory cells and neovasculature in an edematous matrix consisting of residue fibrin, glycoproteins, collagen and glycosaminoglycans (Clark 1985). As the proliferative phase proceeds there is a rapid increase in the tensile strength of the wound, as a scaffolding of collagen is laid down progressively.

1.1.3.5 Stage V: The Maturation Phase (24 days-1 year)

During this phase there is a progressive decrease in vascularity of the scar, shrinkage of the fibroblast, enlargement and reorientation of the collagen fibres and an increase in tensile strength. While the strength of the wound increases rapidly from the 6th to the 21st day, only 50% of the normal tensile strength of a skin wound is regained within the first 6 weeks, and in all healing tissues the amount of collagen in the scar increases for several months.

Contraction and epithelisation of the wounded tissue occurs during the Maturation Phase. Contraction is the process by which large wounds become small without the need for secondary closure or skin graft. The mechanism of wound contraction is not clearly understood, but myofibroblasts, a special type of contractile cell, are involved (Powell 1999). Contraction begins on the 4th day and proceeds side by side with epithelisation and the cellular and biochemical process of wound healing.

Epithelisation is often regarded as the final step in the healing process (Goslen 1988). The process of epithelial resurfacing is a critical part of the wound healing scenario. Skin has a covering of epithelial cells, called keratinocytes. When there is a defect in this covering, these cells migrate towards the area of deficit. This migration begins within hours of wounding during the inflammatory period and is a direct event that does not require a preliminary increase in cellular proliferation (Slavkin 2000).

1.1.4 Growth Factors

Although the sequence of histologic changes in healing wounds has been known for many years, the molecular factors that regulate these processes *in vivo* are poorly understood. Peptide growth factors (or cytokines) appear to play key roles in initiating the phases of tissue repair (reviewed by Slavin 1996; Pierce & Mustoe 1995). Growth factors are soluble proteins or glycoproteins that regulate cell proliferation and induce the migration of cells as well as controlling various processes involved in tissue repair (Robson 1991). They are synthesised and secreted by many types of cells involved in tissue repair including platelets, inflammatory cells, fibroblasts, epithelial cells and vascular endothelial cells. The growth factors may act on the producer cell (autocrine stimulation), adjacent cells (paracrine stimulation) or distant cells (endocrine stimulation; Steenfos 1994). All peptide growth factors initiate their effects by binding to and activating specific high-affinity receptor proteins located in the plasma membrane of target cells. Activation of the receptors eventually results in stimulating a number of processes, including those involved in wound

healing (Bennett & Schultz 1993). There are five major growth factor families that appear to contribute significantly to the healing process (Mutsaers *et al.*, 1997): epidermal growth factor (EGF; Nanney & King 1996), transforming growth factor-beta (TGF-β; Sporn & Roberts 1993; Bruijn *et al.*, 1994; O'Kane & Ferguson 1997), insulin-like growth factor (IGF; Lund 1994), platelet-derived growth factor (PDGF; Heldin & Westermark 1999), Fibroblast growth factor (FGF; Slavin 1995). It has been shown that in wounds of diabetic patients, which heal very slowly, or not at all, levels of growth factor and their receptors are reduced (Braddock *et al.*, 1999).

1.1.5 Scarless Healing

In almost every organ and tissue, scarring following injury or trauma is a major problem. Skin wounds made in early embryos/fetuses heal without scarring. One critical difference between scarfree embryonic healing and scar forming adult healing is the inflammatory response. Embryos mount a poor inflammatory response to wounding with the recruitment of only a small number of inflammatory cells (Ferguson 1998).

It is the level of TGFβ3 on the one hand and TGFβ1 and TGFβ2 on the other, which is important for scar formation. Decreasing the levels of TGFβ1 and TGFβ2 relative to TGFβ3 appears to be the key to the anti-scarring therapy (Ferguson 1998).

1.1.6 Wound Healing and the Extracellular Matrix (ECM)

An intimate involvement of the ECM in all aspects of tissue remodelling and wound healing has expelled the historic notion of the ECM being nothing more than an inert scaffold around cells. Recent observations suggest that the ECM far from being inactive in the wound healing process, actively orchestrates the key steps in the programme of wound healing and regeneration (Figure 1.1; Raghow 1994).

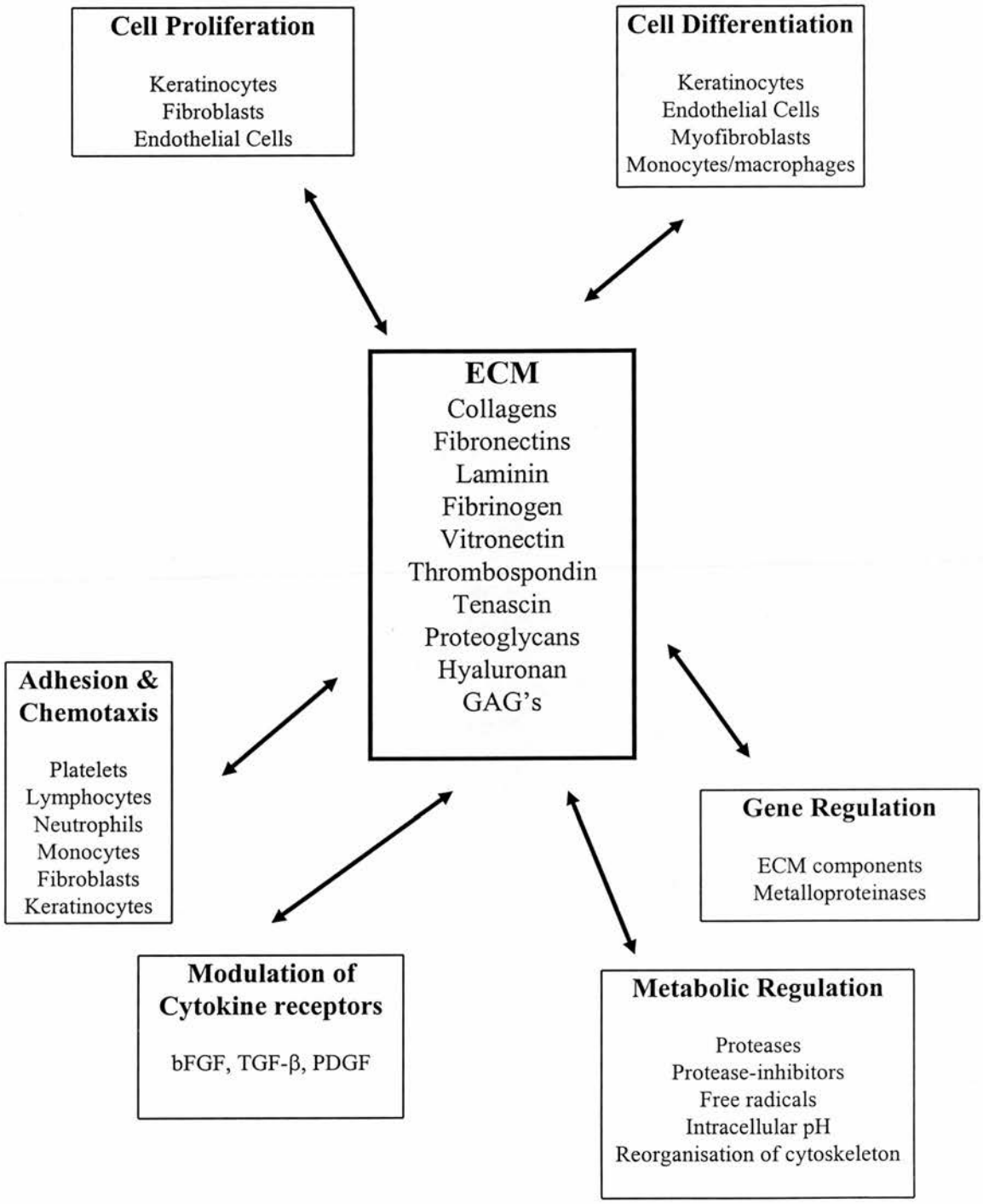

Figure 1.1: Cell-matrix interaction modulate many mechanisms in wound healing (from Raghow, 1994)

1.2 EXTRACELLULAR MATRIX

1.2.1 Introduction

Connective tissue is a multiphase, supporting, interstitial material present throughout mammalian organs and parts. This tissue joins the other tissues of the body together. Connective tissue takes the stress and strain of movement, maintains shape, and can be considered a composite of insoluble fibres and soluble polymers (Ayad *et al.*, 1994). It is comprised of cells, intercellular fibres, intervening matrix materials and interstitial fluid. Interactions between cells and extracellular matrix are essential for normal tissue homeostasis and also in wound healing, though the mechanisms involved are still poorly defined.

The principal fibres in the ECM are collagen and elastin, while the soluble molecules include proteoglycans and glycoproteins (Labat-Robert *et al.*, 1990; Piez 1997). The structure (and hence function) of any connective tissue depends on the relative proportions and organisation of these constituent molecules. Those tissues which have to withstand large tensional force (such as tendon) tend to be particularly rich in fibrillar collagens, while a tissue that has to withstand compressive forces (such as cartilage) contains high levels of proteoglycans.

1.2.2 Elastin

Elastin is the major protein of the elastic fibres that form a randomly orientated, interconnected network in many tissues (reviewed by Rosenbloom *et al.*, 1991; Mecham 1999). Elastin content may vary from 2% of dry weight in skin to over 70% in the nuchal ligament of grazing animals. A high content of hydrophobic amino acids makes elastin one of the most chemically-resistant and proteinase-resistant proteins in the body (Landeau *et al.*, 1994). Its principal function is to provide elasticity and resilience to tissue (Oxlund *et al.*, 1988). Tissues whose function demands elasticity

include elastic cartilage, skin, ligaments, lung, intestine, and blood vessels, particularly the thoracic aorta. Elastin levels fall with increasing age. This loss of elasticity causes increasing rigidity of the arterial wall, leading to functional decline of the cardiovascular system (Robert 1996).

Elastin synthesis has been documented in fibroblasts, smooth muscle cells, chondrocytes and endothelial cells (Mecham *et al.*, 1981; Burke & Ross 1979; Quintarelli 1979; Cantor 1980). Elastin is difficult to extract from tissue because of its insolubility (Sandberg *et al.*, 1969). The soluble precursor of mature elastin, tropoelastin (72kD) has a distinctive composition, being rich in hydrophobic amino acids (Vrhovski & Weiss 1998). Thus glycine (Gly), proline (Pro), alanine (Ala), valine (Val), phenylalanine (Phe), isoleucine (Ile) and leucine (Leu) predominate while aspartic acid (Asp), glutamic acid (Glu), lysine (Lys) and arginine (Arg) make up less than 5%. Elastin is cross-linked through desmosine and isodesmosine residues (Partridge *et al.*, 1963, Thomas *et al.*, 1963). Glycine constitutes one-third of all amino acid residues, but there is no regularly repeated sequence (Gly-X-Y) as in fibrillar collagen. In tropoelastin, hydroxyproline (Hyp) constitutes about 1% of all residues, but tryptophan (Trp), histidine (His), methionine (Met) and hydroxylysine (Hyl) are absent and there is no attached carbohydrate (Mecham 1999). The human tropoelastin molecule consists of 786 amino acid residues, and its complete primary structure is known (Indik *et al.*, 1987). It contains repeated hydrophobic sequences such as Val-Pro-Gly-Val-Gly and Pro-Gly-Val-Gly-Val-Ala, which produce a β-spiral conformation, a structure not found in other mammalian proteins (Rosenbloom *et al.*, 1993). There are also α-helical segments that include eight Ala-rich sequences in which two or three residues are preceded by a sequence of between three and nine Ala residues. These peptides are the site of cross-linking, a process that transforms tropoelastin into insoluble elastin (Bailey & Light 1989).

1.2.2.1 Elastin Degradation

In normal tissues, degradation occurs in the course of very slow elastin turnover (Murphy *et al.*, 1990). Elastin is degraded by elastases isolated from polymorphs, macrophages, platelets and the pancreas (Werb *et al.*, 1982). However, in chronic wounds, the continued presence of neutrophils and macrophages results in a high level of elastase production (Kim & Kang 2000). Elastases are serine proteases specific for small hydrophobic amino acid residues such as Val, Leu and Ile. Elastases are therefore not specific for elastin; they are also capable of degrading type III and IV collagens, fibronectin and proteoglycans. These enzymes can hydrolyse insoluble elastin fibres, usually hydrolysing peptide bonds at the carbonyl side of Gly, Ala or Val residues. Once soluble elastin fragments are generated, hydrolysis can be completed by other neutral proteases like stromelysin and gelatinase. The activity of elastases can be controlled by inhibitors such as α1-antitrypsin and α2-macroglobulin (Hood *et al.*, 1993).

1.2.2.2 Biological properties

The presence of an elastin receptor on several cell types confers to elastin peptides several biological functions: chemotactic activity on monocytes, fibroblasts and smooth muscle cells (SMC), stimulation of elastin fibre adhesion to vascular SMCs and fibroblasts, release of lytic enzymes and oxygen free radicals from leukocytes, cellular proliferation and modulation of vascular tone (reviewed by Fulop *et al.*, 1998).

Elastin is chemotactic towards fibroblasts, both in its precursor form of tropoelastin and in its fragments (Senior *et al.*, 1982). Even though the desmosine cross-link is not required for elastin-dependent chemotaxis (Postlethwaite *et al.*, 1978; Hunninghake *et al.*, 1981), this is also a chemoattractant to fibroblasts (Kunitomo & Jay 1985). Hydrophobic repeating peptides of elastin are also able to generate directed cell

movement in human monocytes and bovine ligamentum nuchae fibroblasts where the latter are capable of synthesising elastin (Senior *et al.*, 1984). Elastin-like synthetic nonapeptides are chemoattractants for bovine aortic endothelial cells (Long *et al.*, 1989). Pro-Gly-Ala-Ile-Pro-Gly, a repeating hexapeptide of both bovine and human tropoelastin, is chemotactic for neutrophils and tumour cells as well as for fibroblasts (Grosso & Scott 1993).

The hexapeptide VGVAPG has been found to be chemotactic towards monocytes (Bisaccia *et al.*, 1994) and induced cellular proliferation (Wachi *et al.*, 1995). It is also thought that this peptide generated at the site of proteolysis during vascular injury may have angiogenic activity (Tummalapalli & Tyagi 1999).

1.2.3 Glycoproteins

Glycoproteins (Heinegard 1986, Brockenhausen *et al*, 1998) are widely distributed in the body. Most plasma proteins, with the notable exception of albumin, are glycoproteins. Glycoproteins are important components of the mucus of the gastrointestinal, respiratory and reproductive tracts. A carbohydrate content of up to 85% is important in endowing these glycoproteins with their mucoid properties. Cell-surface glycoproteins are integral proteins which float on the lipid bilayer and expose their oligosaccharides exclusively on the external surface of the cell. Plasma membrane glycoproteins, together with glycolipids, are crucial to cell recognition phenomena. Glycoproteins consist of one or more oligosaccharide units attached covalently to a protein core (Kobata 1992). Proteoglycans are thus a particular form of glycoprotein, and collagens are glycoproteins because of their glucose-galactose moieties (section 1.3). Typical monosaccharide constituents are N-acetlyglucosamine (GlcNAc), mannose (Man), galactose (Gal), fructose (Fuc), sialic acid, occasionally N-acetylgalactosamine (GalNAc) and rarely, glucuronic acid (GlA). Fuc and sialic acid always occupy non-reducing positions in the oligosaccharide, and Gal is often the next sugar residue.

Several multidomain ECM glycoproteins have been characterised. These include fibronectin, laminin, vitronectin, thrombospondin, tenascin and nidogen (Yamada 1991). Fibronectin and laminin are the major glycoproteins involved in wound healing.

1.2.3.1 Fibronectin

The term fibronectin (Carson 1990; Romberger 1997; Hynes 1999) has been given to a family of glycoproteins found on cell surfaces, and in most extracellular matrices and basement membranes. The functions of fibronectin include roles in cell adhesion and migration, cytoskeletal organisation, embryological development and morphogenesis, haemostasis, thrombosis, wound healing and malignant transformation (Potts & Campbell 1994).

Fibronectin has a molecular weight of 440kDa, and consists of two subunits of molecular weight 220kDa connected by disulphide bonds. Fibronectin contains 4% carbohydrate as four to six oligosaccharide units connected to a protein core via Asn linkages. The main monosaccharide components are Man, Gal, GlcN and sialic acid with lesser amounts of Fuc and possibly Glc (Mosher 1993).

In each fibronectin subunit, the protein is organised in tightly folded globular domains, resistant to proteolytic attack, and connected by regions of polypeptide susceptible to proteolysis. Controlled digestion by proteases therefore 'dissects' fibronectin into its constituent globular domains, which can then be separated to allow study of their individual properties. Domains with binding sites for collagens, gelatin, heparin, cell surfaces, fibrin and bacteria are now recognised (Romberger 1997). A 30kDa domain at the amino terminus binds to fibrin, heparin, actin and *S. aureus* cells. Next is a 40kDa domain that binds to gelatin and native collagens. The cell-binding domain (15kDa) is within a 75kDa region. There are second domains for heparin and fibrin binding next to this (Yamada *et al.*, 1982). Within the domains, three types of short

amino acid sequence (namely types I, II and III) are repeated many times, together accounting for some 95% of the total sequence of fibronectin. The type I and type II sequences, each of about 50 amino acid residues, are repeated 12 times and twice respectively, in each fibronectin subunit, while the type III sequence, of 90-95 amino acids, is repeated 15-17 times (Hynes 1999). Fibronectin type III repeats are particularly widespread, being found in many ECM proteins, cell adhesion proteins and cell surface receptors.

The *S.aureus* binding site is important in disease. *S.aureus* is a natural inhabitant of mammalian skin and being an opportunistic pathogen is a major cause of serious infections especially in the hospital environment. The fibronectin-binding site of *S.aureus* is involved in binding to fibronectin allowing bacterial colonisation and initiation of disease (Brennan *et al.*, 1999).

Fibronectin binds equally well to denatured collagen types I, II and III but less strongly to type V. Collagens have several binding sites for fibronectin (Lapiere *et al.*, 1994). Fibronectin also interacts with cell surface proteoglycans (Engel 1991), hyaluronan (Nakamura *et al.*, 1994), heparan sulphate (Drake *et al.*, 1992) and heparin (Busby *et al.*, 1995).

Fibronectin and fibronectin fragments, from degradation by proteinases from polymorphonuclear leukocytes, are chemotactic for monocytes, polymorphs, fibroblasts and endothelial cells. The release of fibronectin from these cells enhances their adherence at the inflammatory site. In wound healing (Welch *et al.*, 1990), fibronectin promotes migration of keratinocytes (Peltonen *et al.*, 1989), platelet adhesion (Booyse *et al.*, 1982) and stabilisation of the clot. Neutrophil motility, chemotaxis (Jarstrand *et al.*, 1982), and adhesion to endothelial cells (Wall *et al.*, 1982) or material surfaces (Barber *et al.*, 1978) are promoted by fibronectin.

Synthetic peptides derived from fibronectin sequences have become very useful tools. They have been used to modulate cell migration and tumor metastasis *in vivo* and to interfere with platelet aggregation (Davies *et al.*, 1994). Fibronectin-derived peptides have also been used to block lymphocyte attachment in rheumatoid arthritis tissues (Elices *et al.*, 1994) and to attenuate chronic inflammatory changes in bacterial-induced arthritis (Wahl *et al*, 1994).

The appearance of fibronectin in tissues prior to and during cell migration, assists in the direction of cell differentiation and morphogenetic movement (Dufour 1988). Fibronectin appears in the migratory pathways of neural crest cells.

Fibronectin appears to be important in both monocyte migration and phagocytosis. It has been reported that fibronectin promotes monocyte adhesion to material surfaces (Bevilacqua *et al.*, 1981), and both the intact molecule (Yonemasu *et al.*, 1983) as well as fibronectin fragments (Norris *et al.*, 1982) were found to be chemotactic for monocytes.

Fibronectin enhances the opsonin-independent pathway of phagocytosis by monocytes (Ginsberg 1979). Fibronectin also stimulates secretion of monocyte derived growth factor for fibroblasts (Martin *et al.*, 1983). Adhesion of fibroblasts to material surfaces (Grinnell 1978) and denatured collagen (Kleinman *et al.*, 1981) is promoted by fibronectin. This glycoprotein has been found to be chemotactic for fibroblasts (Postlethwaite *et al.*, 1981), suggesting that fibronectin is very important for fibroblast migration into the wound bed. This chemotactic activity has been shown to be restricted to a defined region of the fibronectin molecule which is the same for various fibroblast strains. The active domain is localised between the collagen binding site and the major heparin binding site, about 170kDa from the N-terminal and about 70kDa from the C-terminal ends of the two subunit peptide chains (Albini *et al.*, 1983).

Fibronectin may also be essential in the organisation of the granulation tissue matrix, as *in vitro* studies have shown that collagen, heparan sulphate proteoglycan and chondroitin sulphate are co-deposited with fibronectin (Hedman *et al.*, 1982). Endothelial cell adhesion (Macarak & Howard, 1983) and chemotaxis (Bowersox & Sorgente 1982) are promoted by fibronectin. It is also an attachment and spreading factor for keratinocytes (Gilcrest *et al.*, 1982; Stenn *et al.*, 1983). Fibronectin-coated material surfaces provide a suitable substratum for epidermal cell migration *in situ* (Donaldson *et al.*, 1983). These results suggest that fibronectin could be the substrate for keratinocyte migration during wound healing. Fibronectin has been shown to promote the phagocytosis of latex particles by keratinocytes (Takashima & Grinnell 1985). Finally, the reorganisation of the basement membrane beneath the epidermal cells after their migratory activities have ceased may be promoted by fibronectin (Brownell *et al.*, 1981). Fibronectin is encoded by one single gene, and diversity is generated by alternative splicing of the primary transcript (Shwarzbauer *et al.*, 1983). Different forms of fibronectin are expressed at the wound site (alternate splicing). Wounding activates fibronectin synthesis in the underlying and surrounding dermis and this fibronectin is of the splice type commonly seen in embryonic situations associated with proliferation and migration (Hynes 1989).

1.2.3.2 Laminins

Laminins are a family of large glycoproteins that are distributed ubiquitously in basement membranes (reviewed by Engel 1992; Tryggvason 1993; Ekblom *et al.*, 1998). These molecules are multifunctional, performing key roles in development, differentiation and migration. They interact with cells via cell-surface receptors and with other basement membrane components such as type IV collagen, entactin/nidogen and heparan sulphate proteoglycan (Tanzer *et al.*, 1993). Laminins represent a protein family of α, β, chain heterotrimers primarily located in basement membranes but also in some mesenchymal components. So far 11 different chains have been identified giving rise to laminins 1-11 (Sasaki & Timpl 1999).

Like fibronectin, laminin can influence cell adhesion, growth, morphology, differentiation, migration, and agglutination as well as the assembly of the extracellular matrix (Tanzer *et al.*, 1993). By limited cleavage with elastase, chymotrypsin, pepsin or cathepsin, laminin can be cleaved into biologically active fragments (Aumailley *et al.*, 1987; Hershkoviz *et al.*, 1995). Laminin primarily affects cells of epithelial origin, and the response varies depending on the cell. It binds to various components of the basement membrane and probably links these to one and other (Terranova 1980) to form an integrated complex (Dziadek 1995). Laminin promotes haptotactic migration of murine B16 melanoma cells (McCarthy & Furcht 1984) as well as being chemotactic for PMN (Terranova *et al.*, 1986) and for bronchial epithelial cells (Rickard *et al.*, 1993).

1.2.4 Proteoglycans

Proteoglycans (reviewed by Kjellen & Lindahl 1991; Hardingham & Fosang 1992; Lander 1999) are complex macromolecules that contain a core protein to which at least one glycosaminoglycan (GAG) chain is covalently bound. This simple definition encompasses a wide range of structures involving different core proteins, different classes of GAG, and different numbers and lengths of GAG chain. This enormous versatility at molecular level allows proteoglycans to serve many diverse structural and organisational functions in tissues. These diverse groups of heterogeneous macromolecules are found in almost all mammalian tissues and are especially prominent in connective tissues (Kuettner & Kimura 1985).

Glycosaminoglycans are linear anionic polysaccharides with repeating disaccharide units containing a hexosamine residue and usually, but not always, a hexuronic acid residue. With the exception of hyaluronic acid, they are almost always sulphated. The GAGs can be divided into four classes: chondroitin and dermatan sulphates, keratan sulphates, heparan sulphates and heparin, and finally hyaluronic acid. Each

proteoglycan has a trivial name that refers either to biological activity (eg decorin, which binds to and decorates the surface of collagen fibrils; Fleischmajer *et al.*, 1991) or to sequence (e.g. serglycin, which contains a large alternating sequence of serine and glycine residues; Avraham *et al.*, 1989). Proteoglycans can be either secreted and deposited in the ECM (e.g. aggrecan, versican, biglycan, decorin, fibromodulin; Esko 1991) or located intracellularly in secretory glands (e.g. serglycin; Avraham *et al.*, 1989). The most extensively studied proteoglycans are those from mammalian cartilages. Proteoglycans are central to the proper functioning of cartilage, which is to be able to reversibly absorb loading forces (Muir 1983).

The breakdown and degradation of proteoglycans has been extensively studied, primarily in relation to cartilage degradation (Buttle *et al.*, 1993). Neutral proteinases have been identified that act on the protein core, the initial cleavage probably occurring near the hyaluronate binding region of the protein core. The GAG side chains can be degraded by glycosidases and the hexoses cleaved off by specific iduronidases and glucuronidases.

The extreme variety of proteoglycans is associated with a wide range of functions (Bailey & Light 1989), of which many are mediated by interactions of proteoglycans with other molecules. Proteoglycans can act as space filling molecules which limit diffusion of other molecules through the tissue. Dermatan sulphate proteoglycans control rigidity and flexibility of the tissue matrix (Kresse *et al.*, 1994).

Proteoglycans are known to be important in several physiological aspects of wound healing. Heparan sulphate proteoglycans, which are located in basement membranes (BM), are associated with cell membrane receptors and may be involved in cell matrix interactions (Ihrcke *et al.*, 1993). Such interactions may be important in cell adhesion (Wight *et al.*, 1992). It is becoming clear that heparan sulphate proteoglycans have an important role to play in tissue repair (McGrath & Eady 1997). The findings of Andriessen *et al.*, 1997, suggest that their levels vary with time after healing,

suggesting a correlation between normalisation of epidermal proliferation, BM permeability and regeneration of BM heparan sulphate.

Hyaluronic acid (HA), also known as hyaluronan, is one of the most hydrophilic molecules in nature and this characteristic is probably the key contibutor to many of its biological functions (reviewed by Chen & Abatangelo 1999). Hyaluronic acid is the principal glycosaminoglycan present in fetal wounds and is thought to contribute to the scarless healing of wounds (Adzick & Longaker 1992). HA has also been shown to play a role in the inflammatory phase of wound healing (Kobayashi & Terao 1997), as well as being involved in angiogenesis (Noble *et al.*, 1998) and re-epithelisation (Tuhkanen *et al.*, 1998). A HA-rich matrix is permissive for cell motility (Toole 1997) and proliferation (Adzick & Lorenz 1994). West *et al.*, 1997, have shown that in adult and late gestation fetal wound healing, removal of hyaluronan results in fibrotic scarring. A hyaluronan-rich matrix may reduce collagen deposition, leading to reduced scarring.

The breakdown products of HA have been reported to have biological activities. *In vivo* degradation of HA within fetal rabbit wounds results in an altered healing response such that there is a marked increase in fibroplasia, collagen deposition and neovascularisation (Mast *et al,* 1994). Partially cleaved HA also promotes angiogenesis *in vivo* and *in vitro* and stimulates migration and proliferation of normal endothelial cells (Kumar *et al.*, 1992).

1.3 COLLAGEN

1.3.1 Introduction

The word collagen derives from the Greek Kolla, meaning glue, and the term 'glue-former' or 'collagen' was originally used in the 19th century for the component in skin, bone, cartilage and tendon, which when the tissues were boiled in water and the extracts evaporated to produce glue (Weiss & Ayad 1982). The economic importance of collagen has been recognised for thousands of years. Collagen, the main constituent of skin (review by Olsen & Ninomiya 1999), is also the raw material of leather and gelatin, which have long been commercially important medical commodities (Kielty *et al.*, 1993).

Collagens are proteins present throughout the animal world. Most of the body scaffolding in mammals is composed of collagen (Burgeson 1988; Fleishmajer 1990) which provides the essential framework of connective tissues (Kuhn 1986, Nimni 1988). Many cells lie on a collagenous basal lamina or exist within a collagenous matrix. Cell-collagen interactions are essential to cell movements in inflammation, wound healing, trophoblast implantation, fetal development and cancer (Minafra *et al.*, 1995).

Collagen itself is a glycoprotein, unique in this classification since it contains no amino sugars and in fact has only two types of carbohydrate residues - glucose and galactose - in its structure. It is a large extracellular protein which is composed of three polypeptide chains (called α-chains) each of which contains long sequences of repeating tripeptides based on the general structure Gly-X-Y (where X is commonly proline and Y can represent any amino acid but is often the modified amino acid hydroxyproline; van der Rest & Garrone 1991). All collagens also contain varying quantities of the amino acid hydroxylysine which is formed by the enzyme-catalysed hydroxylation of lysine residues in newly synthesised α-chains (Bailey & Light 1989).

Because of this primary sequence the collagen polypeptide α-chain adopts a left handed polyproline type triple helix (Ramachandran & Kartha 1954, 1955; Ramachandran & Reddi 1976; Ramachandran 1988). Each chain assembles with two other chains in right handed superhelix in which every glycine residue is buried along the axis of the helix (Traub & Piez 1971, Piez & Miller 1974).

1.3.2 Collagen Types

Collagen, known for its versatility, is found in many different parts of the body, taking different forms to fulfil various functions, which may explain why so many different types of collagen are found (Prockop & Kivirikklo 1995). As the number of collagen types defined in the literature has increased (19 to date; Ricard-Blum *et al.*, 2000), it has been considered appropriate to attempt to discuss the collagens in terms of groups or classes. The collagen numbering system (Roman numerals for each collagen type and Arabic numerals for individual α-chains) to some extent reflects the relative abundance of the various collagens, in that generally the more abundant collagens were identified earliest.

Collagen types form a wide range of structures (van der Rest & Garrone 1991; Prockop & Kivirikklo 1995; Tryggvson 1995; Olsen & Ninamiya 1999). Most notable are: i) fibrils that are found in most connective tissues and which are made of alloys of fibrillar collagens (types I, II, III, V and XI) and ii) sheets constituting basement membranes (type IV collagens), Descemets' membrane (type VIII collagen), worm cuticle, and the organic exoskeleton of sponges (Scott 1995).

Other collagens, present in smaller quantities in tissues, play the role of connecting elements between these major structures and other tissue components. Collagens known as FACIT (fibril associated collagens with interrupted triple helix), such as types IX, XII, XIV, XVI, XVIII and XIX associate with the surface of fibrils and

modify their interactive properties (Olsen 1989; Ricard-Blum *et al.*, 2000). Type VII collagen assembles into anchoring fibrils that bind epithelial basement membranes and entrap collagen fibrils from the underlying stroma to glue the two structures together (Burgeson 1993). Type VI collagen forms thin-beaded filaments that may interact with fibrils and cells (Timpl & Chu 1994). The molecular configuration, supramolecular structure and tissue distribution for types I-XIX collagens are listed in Table 1.1.

In terms of the whole body collagens, types I, II and III are quantitatively the most important and are generally considered to account for over 70% of the total (Kuhn 1987). These collagen types constitute the classical fibril-forming collagens. They are synthesised intracellularly as large precursor procollagens comprising a continuous triple helix, at each end of which are non-helical domains (propeptides). The propeptides are cleaved extracellularly by specific N- and C-proteinases during fibrillogenesis giving rise to the collagen monomers consisting of the triple helix flanked by non-helical regions. The monomers spontaneously assemble to form the fibril, each monomer staggered by 234 amino acid residues. This results in maximal electrostatic and hydrophobic interactions between adjacent monomers and allows specific lysine/hydroxylysine residues in the helix and non-helical regions to form stable covalent cross-links (Bishop & Laurent 1995).

A collagen triple helix has a relatively high thermal stability and it is resistant to digestion by the majority of proteases in the extracellular matrix (Murphy & Reynolds 1993). The stability of the triple helix is provided by interchain hydrogen bonds between the amide of glycine and the carbonyl oxygen of an X-position residue on an adjacent chain (Prockop & Kivirikklo 1995). Additional hydrogen bonds that involve the hydroxyl group of hydroxyproline, a water molecule and a backbone carbonyl further stabilise the molecule (Klein & Huang 1999). Thus in the absence of hydroxylation the triple helix of type I collagen denatures at about 27°C (Brown *et al.*, 1994), but with complex hydroxylation the denaturation temperature is about

Table 1.1: Vertebrate collagens

Type	α chain(s)	Supramolecular structure	Tissue distribution: some examples
I	α1(I), α2(I)	Fibrillar	Bone, skin, cornea, lung, tendon
II	α1(II)	Fibrillar	Cartilage, vitreous humour
III	α1(III)	Fibrillar	Skin, lung, vascular system,
IV	α1(IV), α2(IV), α3(IV), α4(IV), α5(IV)	Network	Basement membrane
V	α1(V), α2(V), α3(V)	Fibrillar	Collagen I containing tissue
VI	α1(VI), α2(VI), α3(VI)	'Beaded' microfibrils	Most connective tissues
VII	α1(VII)	Anchoring fibrils	Basement membrane (skin, oral mucosa, cervix)
VIII	α1(VIII), α2(VIII)	Hexagonal arrays	Descemet's membrane, endothelial cells
IX	α1(IX), α2(IX), α3(IX)	Surface of collagen II fibrils	Collagen II containing tissues
X	α1(X)	Hexagonal arrays	Hypertrophic and mineralising zones of cartilage
XI	α1(XI), α2(XI), α3(XI)	Fibrillar, core of collagen II fibrils	Collagen II containing tissue
XII	α1(XII)	? associated with collagen I fibrils	Collagen I containing tissue
XIII	α1(XIII)	? transmembrane	most connective tissues?
XIV	α1(XIV)	? associated with collagen I fibrils	Collagen I containing tissue
XV	α1(XV)	unknown	Widespread
XVI	α1(XVI)	unknown	Human fibroblasts (from cDNA)
XVII	α1(XVII)	transmembrane	Human skin (from cDNA)
XVIII	α1(XVIII)	unknown	Widespread, most abundant in liver and lung
XIX	α1(XIX)	unknown	Basement membrane (liver, kidney, spleen)

References: Types I-XIV (Hulmes, 1992; Brown and Timpl, 1994), Types XV-XVIII (Mayne and Brewton 1993), Types XV, XVII and XVIII (Pihlajameini and Rehn, 1995), Type XIX (Myers *et al.*, 1997), Types VI, VII, VIII, IX, X, XIV, XVI, XIX (Richard-Blum *et al.*, 2000).

42°C. It is likely that other factors, especially charge-to-charge interactions, contribute to the stability of the triple helix; although these factors have been recognised for some time, their importance is often overlooked (Naito *et al.*, 1994). Once denatured, the sensitivity of the chains to most proteases ensures normal turnover.

1.3.3 Biological Properties

Apart from mechanical strength and ability to bind other matrix components, collagens show additional important properties, these are described below.

1.3.3.1 Cell Attachment and Proliferation

Many cell types, including human fibroblasts, attach *in vivo* to collagen as their natural substratum and bind equally well to types I, II, III or IV (Anderson 1992). It was in 1956 that Ehrmann and Gey made the initial observation that collagen gels enhance the growth of many cell types *in vitro.* The effect of adhesion to the substratum could be direct or be mediated via collagen-bound factors such as fibronectin, laminin or chondronectin (Hay 1991).

The C-terminal propetide of type I collagen is a constituent of bone and is thought to play a role in cell-attachment of osteoblasts (Mizuno *et al.*, 1996).

Type V collagen inhibits proliferation of human endothelial cells (Fukuda *et al.*, 1988). The mechanism remains unknown, but it appears to be related to the detachment of cells. The disassembly of F-actin filaments was observed in cells cultures on Type V (Yamamoto *et al.*, 1992) In general, however, other types of collagen have been found to stimulate endothelial cell (EC) proliferation *in vitro* (Thoumine *et al.*, 1995).

Collagen VI promotes cell attachment, spreading and proliferation (Ruhl *et al.*, 1999).

1.3.3.2 Effect on haemostasis

Exposure of collagen to blood and subsequent aggregation of platelets is important in haemostasis, as is coagulation (Morton *et al.*, 1987; Asselin *et al.*, 1997). Adhesion of platelets to collagen fibres triggers release from platelet granules of serotonin, adrenaline, adenosine diphosphate and thromboxane A_2 which stimulate platelet aggregation. Collagen may also activate the intrinsic coagulation pathway either directly or indirectly, by release of, or exposure to, coagulation factors bound to platelets. The ability to induce platelet aggregation seems to be a property of tertiary and quartenary structure rather than that of collagen type and the adhesive process is mediated by von Willebrand factor (Fitzimmons *et al.*, 1988). Integrin $\alpha 2\beta 1$ mediates adhesion to collagen, thereby allowing platelet interaction with a lower affinity receptor (Alberio & Dale 1999).

1.3.3.3 Collagen-derived peptide activities

Types I, II and III collagens and their derived-peptides have been shown to be chemotactic towards a variety of cell types. Over the years several groups have studied collagen peptides and their activities, conflicting results being found in many cases (Postlethwaite *et al.*, 1976. 1978; Albini & Adelmann-Grill 1985; Malone *et al.*, 1991).

The degradation products of collagen, released from necrotic tissue, were first shown to be involved in neutrophil infiltration by (1971). Recent work by Thomas *et al.*, 1999, suggests that necrotic tissue is composed partly of low molecular weight ECM proteins, representing the degradation products of autolytic debridement. Postlethwaite & Kang (1976) followed this initial discovery of Houck & Chang by showing that native tropocollagen and its peptide fragments, derived by degradation

with cyanogen bromide (CNBr), pepsin and also bacterial collagenase, were all chemotactic for monocytes. On the other hand, neutrophils did not recognise any of these as chemotactic stimuli. Collagen peptides as small as three amino acids long were shown to be chemotactic for monocytes (Postlethwaite & Kang 1976). The size of collagen fragments is not constant as the molecule undergoes degradation by non-specific proteases, resulting in peptide of various sizes (Murphy & Reynolds 1993).

Native collagen and its constituent α chains are chemotactic towards fibroblasts (Postlethwaite *et al.*, 1978). Collagen types I, II, and III after digestion with bacterial collagenase, are all fibroblast chemoattractants. The synthetic di and tri peptides Gly-Pro-Hyp, Pro-Hyp and Gly-Phe-Ala are also chemotactic for fibroblast cells, though the individual amino acids are not (Postlethwaite *et al.*, 1978). In contrast only collagen peptides formed after cleavage with mammalian collagenase were chemoattractants for fibroblasts. Contradictory to this past evidence (Postlethwaite *et al.*, 1978), bacterial collagenase abolished this activity (Albini & Adelmann-Grill 1985).

Collagen peptides, both native and synthetic, have been shown to induce a neutrophil influx in rats (Riley *et al.*, 1984). Collagen digested with bacterial collagenase and CNBr-generated-peptides were found to be potent chemoattractants for neutrophils. The synthetic peptides $(Pro\text{-}Pro\text{-}Gly)_5$ and $(Pro\text{-}Hyp\text{-}Gly)_5$ produce a maximal induction of chemotaxis at 5-10nM (Laskin *et al.*, 1986).

Malone *et al.*, (1991) examined intact Type I collagen, TC^A and TC^B (two characteristic products of collagen generated by mammalian collagenase digestion - see section 1.3.4.2) and Type I gelatin peptides for chemotaxis, using monocytes and neutrophils. Intact Type I collagen was chemotactic for monocytes but not neutrophils.

Most recently, Laskin *et al.*, (1994) found that collagen digested with bacterial collagenase or CNBr, as well as small molecular weight synthetic polypeptides in the

form $(Pro\text{-}Pro\text{-}Gly)_5$ were all potent chemoattractants for rat alveolar macrophages inducing migration in the nanomolar concentration range. These peptides also stimulated the release of superoxide anion and hydrogen peroxide, as well as elastase and gelatinase. Synthetic peptides containing hydroxyproline also stimulated superoxide anion and hydrogen peroxide release, but not chemotaxis. In contrast synthetic peptides containing Pro and Gly induced migration of macrophages. Peptides of the form $(Pro\text{-}Pro\text{-}Gly)_5$ were active in inducing chemotaxis while longer peptides e.g. $(Pro\text{-}Pro\text{-}Gly)_{10}$ displayed negligible activity. This indicates that peptide length plays a significant role in chemotactic potency and that a critical length may be required.

From all the previous studies, it is thought that the breakdown products of collagen play an active role in the repair of wounded tissue, by attracting cells capable of contributing to repair into the wounded area.

Cyanogen bromide-derived collagen peptides have also been shown to effect the attachment and migration of neural crest cells (Perris *et al.*, 1993), with CB3 from the type I collagen α1 chain promoting greatest cell attachment and migration. CB3, as well as CB6, CB7 and CB8 from the same collagen chain, also support platelet adhesion (Saelman *et al.*, 1993). CB3 and CB4 from the α1 chain of type III collagen also has platelet adhesion activity (Perris *et al.*, 1993). It has been suggested that the sequence Lys-Asp-Gly-Glu-Hyp-Gly may serve as a platelet recognition site in the collagen fragment α1(III) (Morton *et al.*, 1991). CB3 from the α1 chain of type IV collagen participates in cell binding (Vandenberg 1991) but also inhibits proliferation of melanoma cells (Han *et al.*, 1997). Barnes *et al.*, have studied the platelet reactivity of fragments of collagens I and III. α1(III) CB4 was highly aggregatory, α1(I) CB3 only supported platelet adhesion with negligible aggregatory activity. It was thought that the lysyl residues were important for its platelet reactivity. Collagen-platelet interaction involves a basic recognition of the triple helix.

Work by Knight *et al.*, 1999, showed that platelet activation by collagen involves the highly specific recognition of the Gly-Pro-Hyp sequence by platelet glycoprotein VI. Their work demonstrates that a two-step process for collagen-platelet interaction was needed, in which both integrin $\alpha 2\beta 1$ and GpVI are essential.

Collagen peptides from type IV collagen (major collagenous component of basement membranes) have also been shown to be bioactive, promoting cell-adhesion, spreading and motility (Li *et al.*, 1997). Collagen IV also inhibits PMN activation. The inhibitory activity was localised in the non-collagenous domain of the $\alpha 3$ chain containing residues 185-203 (Ziaie *et al.*, 1999). The CD47-$\alpha V\beta 3$ integrin complex was found to be the receptor for the $\alpha 3$(IV) peptide (Shahan *et al.*, 2000).

1.3.4 Collagen Degradation

1.3.4.1 Introduction

The collagen triple helix is highly resistant to the action of most proteinases, but connective tissue cells have been shown to synthesise and secrete a group of proteinases that act on collagen and function under physiological conditions and which are, therefore, important in matrix metabolism. These proteinases can be divided into four classes: aspartic, cysteine, metallo- and serine dependent (Barrett 1980). Collagen degradation (reviewed by Murphy & Reynolds 1990; Birkedal-Hansen *et al.*, 1993; Shingleton *et al.*, 1996) occurs as part of normal tissue turnover. Tissue turnover is increased in development, growth, tissue remodelling, wound healing, some disease processes and also in the involuting uterus and parturient cervix (Woesnner 1991).

Complete collagen degradation is usually the result of the synergistic action of several matrix metalloproteinases (MMPs), also called matrixins (MT), active at neutral pH (Matrisian 1992; Magnatti *et al.*, 1996; Murphy & Knauper 1997; Sternlicht & Werb

1999). These enzymes (see Table 1.2) require both Zn^{2+} and Ca^{2+}, and are secreted in latent proenzyme forms by cells such as fibroblasts, chondrocytes, osteoblasts and endothelial cells (Werb 1982). Collagens are cleaved at specific sites by MMPs (Vankemmelbeke *et al.*, 1998). The breakdown of collagen is summarised in Figure 1.2.

The activity of MMPs is tightly regulated, mainly through the tissue inhibitors of metalloproteinase or TIMPs (reveiwed by Harcharan & McCormick, 1997). To date 4 TIMPs have been described (TIMP-1, 2, 3, 4), each being a separate gene product. The TIMPs function is to regulate the activity by stabilising the proenzyme and by inhibition of the active species (Iredale 1997).

The expression of most MMPs is transcriptionally regulated by growth factors, hormones, inflammatory cytokines, cell-matrix interactions and cellular transformation (Nagase *et al.*, 1999). The equilibrium between synthesis and degradation of ECM components is tightly controlled by MMPs and TIMPs to maintain homeostasis and tissue integrity (Forget *et al.*, 1999). Disruption of this balance may result in diseases associated with uncontrolled proteolysis of connective tissue such as arthritis, tumor cell invasion and metastasis, atherosclerosis and fibrosis (Nagase *et al.*, 1999).

1.3.4.2 Degradative Enzymes

The best characterised proteinase is the mammalian collagenase MMP1 which is a Zn^{2+}-metallo-endopeptidase capable of cleaving the collagen molecule across the three chains to give two triple helical fragments. Gross and Lapiere (1962) first described this enzyme, as it was released into tissue culture medium or tissue explants from tadpole tails undergoing morphogenesis. Fibroblasts, chondrocytes and related cells have been reported to synthesise and secrete collagenase. The enzyme is capable of cleaving all the fibrous collagens, although Type II is cleaved at a slower rate than Types I and III, which are cleaved at approximately the same rate. It has been

Table 1.2
(adpated from Greenwald & Woesnner 1999 and Forget *et al.*, 1999)

MMP Number	Common Name	Substrate
1	Collagenase 1 Fibroblast collagenase Interstitial collagenase	collagens I, II, III, VII, VIII, X; gelatin; aggrecan; tenascin
2	Gelatinase A 72-kDa Gelatinase	laminin; gelatin; fibronectin; collagens I, II, IV, V, VII, X, XI; aggrecan; elastin; vitronectin; type IV collagenase
3	Stromelysin	proteoglycans; laminin; gelatin; fibronectins; collagens I, III, IV, V, IX, XI; aggrecan; link protein; elastin; transin; tenascin; decorin; vitronectin; procollagen peptides; proteoglycanase
4, 5, 6	not used	
7	Matrilysin	proteoglycans; laminin; fibronectin; gelatins; collagen IV; elastin; entactin; tenascin C; aggrecan; link protein; vitonectin
8	Collagenase 2 neutrophil collagenase	collagens I, II, III, VII, VIII, X; gelatin; aggrecan; tenascin
9	Gelatinase B 92-kDa Gelatinase	gelatin; fibronectin; collagens II, IV, V, VII, X, XI; aggrecan; elastin; vitronectin; type V collagenase
10	Stromelysin 2	proteoglycans; laminin; gelatins I, III, IV, V; fibronectin; collagens I, III, IV, V, VIII, IX; aggrecan; elastin
11	Stromelysin 3	N-terminal domain cleaves casein; α1 proteinase inhibitor
12	Macrophage elastase	proteoglycans; laminin-1; elastin; fibronectin; collagen IV; entactin
13	Collagenase 3 Rat osteoblast collagenase	collagens I, II, III, VII, X; aggrecan; gelatin
14	MTI-MMP	activates MMP-2 and -13; laminin B chain; gelatin; fibronectin; collagens I, II, III; aggrecan; tenascin; vitronectin; perlecan; dermatan sulphate; nidogen
15	MT2-MMP	activates MMP-2; laminin; gelatin; fibronectin; collagens I, III; aggrecan; tenascin; perlecan; nidogen
16	MT3-MMP	activates MMP-2; collagens; gelatin
17	MT4-MMP	activates MMP-2; collagens; gelatin
18	Collagenase 4	
19	no trivial name	aggrecan
20	Enamelysin	amelogenin

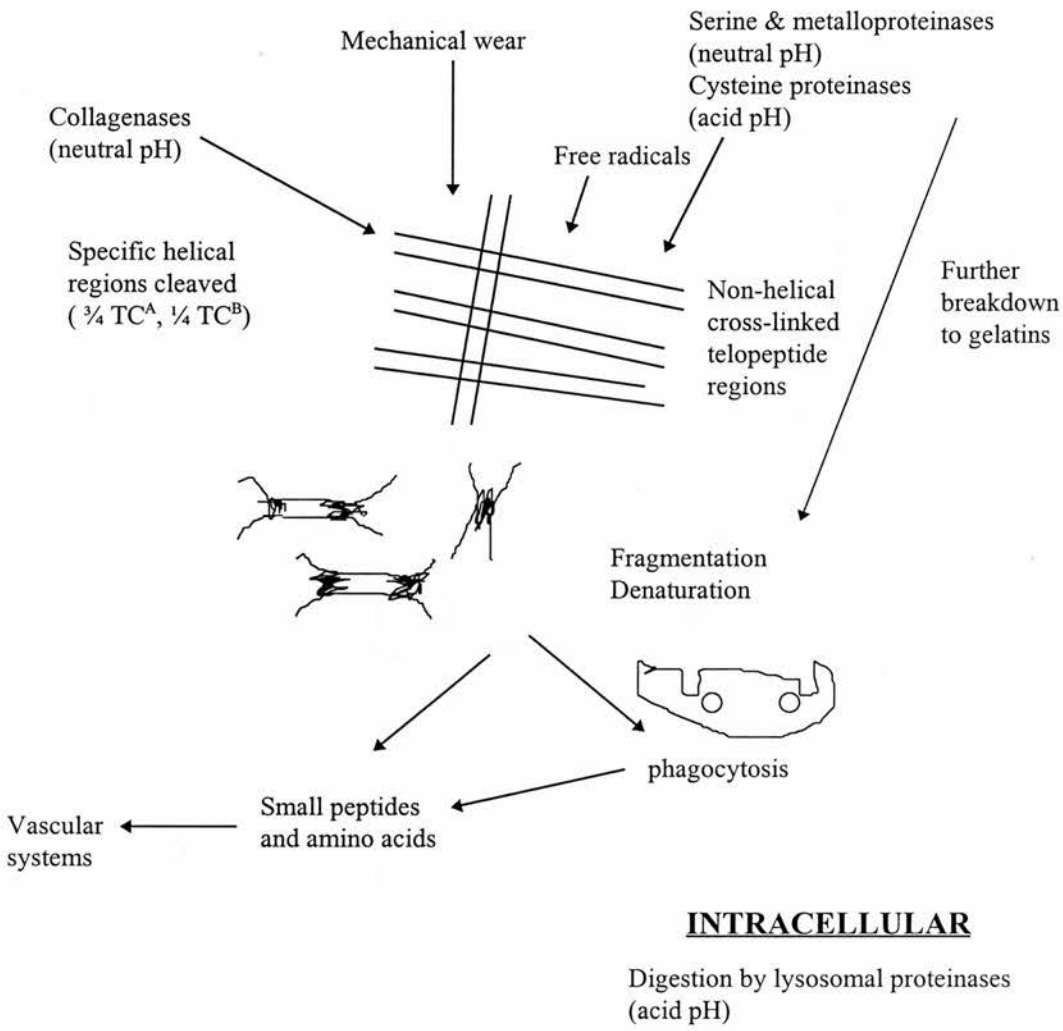

Figure 1.2: Pathways for the degradation of collagen
Initial fragmentation of cross-linked insoluble collagen occurs extracellularly. Cleavage within the collagen triple helix, produces fragments TC^A and TC^B . These fragments denature at 37°C and breakdown further producing small peptides and amino acids (Murphy & Reynolds 1993).

established that the binding of the enzyme is conformationally dependent since it acts on the collagen triple helix at a discrete locus across all three peptide chains, but has little action on the same site on individual α-chains (Fields 1991). Cleavage occurs at the Gly-Ile peptide bonds at residues 775-776 in the α1(I) chain and Gly-Leu in the α2(I) (Highberger *et al.*, 1979). This locus is three-quarters the length of the molecule from the amino terminal end, thus yielding two triple helical fragments, designated TC^A and TC^B (Gross & Nagai 1965). Both fragments retain their triple helical character but have a denaturation temperature below that of the intact molecule ($39^{\circ}C$) and therefore denature at physiological temperatures (Stark & Kuhn 1968). The denatured chains are readily digested by most proteinases, in contrast to the enzymic resistance shown by the triple helix.

Bacterial collagenases were discovered long before their mammalian counterparts. They have been used mostly in laboratories but also as pharmacological agents (Keil 1988). The best characterised bacterial collagenase is produced by *Clostridium histolyticum.* This bacterium produces a group of collagenases with molecular weights ranging from 68 kDa to 125 kDa (Harper 1980). They are metalloproteinases that require zinc and calcium (Bond & Van Wart 1984) which cleave peptide bonds in native, triple-helical collagen (Mandl *et al.*, 1953). The enzyme degrades the helical regions in native collagen preferentially at the Y-Gly bond in the sequence -Pro-Y-Gly-Pro- where Y is most frequently a neutral amino acid, leaving the collagen monomer split into many tripeptide (Gly-X-Y) fragments. *C. histolyticum* -derived collagenase cleaves different collagen types at nearly the same rate (Hatz *et al.*, 1994). It has been suggested that bacterial collagenases may be capable of activating latent mammalian collagenase, thus contributing to the degradation of collagen indirectly (Harrington 1996).

1.4 USES OF COLLAGEN

Collagen, as previously mentioned, has a number of desirable and unique physical and biological properties. Due to these properties, Type I collagen has been used extensively to formulate medical materials (Pachence *et al.*, 1986; DeLustro *et al.*, 1987). Collagen-based medical devices have had a major impact in the field of soft tissue repair. Collagen has been used in medicine as far back as 175 A.D. by Galen who used absorbable cat gut sutures (Katz & Turner 1970). This protein has also been used as a burn dressing; the advantages of collagen dressings were pointed out by Chvapil (1977, 1982). Collagen sponges were shown to cover large wounds, so providing good adherence and protection against mechanical and bacterial infection, whilst preserving fluid and diminishing pain. These dressings have also been developed to function as artificial skin (Yannas & Burke 1980; Murphy *et al.*, 1990). Collagen sponges have also been used as dressings for other wound types such as pressure sores and leg ulcers (Chvapil *et al.*, 1986). The advantages of using collagen sponges for these applications are the formation of new granulation tissue, epithelialisation of the wound, minimisation of contracture and minimum antigenic response. Collagen sponges also produce less inflammation and a lower incidence of infection.

Collagen matrices containing hyaluronate and fibronectin can increase fibroblast proliferation and improve the deposition of organised repair tissue (Doillan *et al.*, 1987, 1988). Chemically modified collagen membranes have been used as drug carriers for the slow release of ophthalmic medication (Miyata *et al.*, 1979). Reconstituted collagen has been successfully used in the treatment of highly vascularised soft tissues such as liver and spleen (Magarit *et al.*, 1987; Sakon *et al.*, 1989). Collagen in an injectable form, zyderm collagen implant (ZCI), has been successfully used as an implant in the management of ageing face, trauma surgery and infection (Matton *et al.*, 1985). Collagen Replacement Therapy is the most common collagen used for replenishment of natural skin collagen. Recently, a new form of

injectable collagen that is prepared from the patient's own tissue has been introduced by Collagenesis Inc. (Beverly, MA, USA). This collagen material named Autologen, persists longer and minimises the risk of allergic reactions (Ruszczak & Schwartz 1999). In the form of fibres, collagen can be used as suture material, and can be inserted into vascular prostheses (Stenzel *et al.*, 1974), blood-vessel prostheses, catheter cuffs and urinary sphincter implants (Ruszczak & Schwartz 1999).

In the last ten years, numerous collagen-based medical devices have been approved, and a few have reached commercial success, see Table 1.3.

One of the reasons that collagen has become the biomaterial of choice for a number of important medical applications is that large quantities of medical grade collagen (collagen, from closed herds, in its purest form) can be easily and cheaply obtained. Also, there are already a number of established collagen products. Collagen has a good safety profile as a biomaterial. Another reason is that collagen can be used in forms that are easily used in minimal invasive procedures. Finally, the understanding of collagen's role in wound healing, metabolism and catabolism, and the interaction between cells and collagen has greatly improved.

Despite these useful properties of collagen, few collagen products have become a commercial success. It is hoped that further advances in the use of collagen will occur in the years ahead. Early efforts using collagen in the form of gels, non cross-linked sponges and films failed to show adequate clinical success. For example, cross-linked collagen films have been shown to function as biocompatible barriers, but have poor oxygen permeability, enhance pooling of wound fluids and ultimately will not support tissue growth (Pachence 1996).

Questions of the antigenicity of collagen have also been raised (Cucin & Barek 1983). Approximately 3% of patients develop a hypersensitivity to injected collagen. A relationship between the use of injectable collagen and the autoimmune diseases

Table 1.3: Commercially Available Collagen-Based Medical Devices
(from Pachence 1996)

MEDICAL SPECIALITY	APPLICATION
General Surgery	Hemostasis
Dermatology	Soft Tissue Augmentation
Dentistry	Oral Wounds Periodontal Ligament Attachment
Ophthalmology	Corneal Shields
Cardiovascular	Anti-infectious Catheter Cuffs Arterial Puncture Repair
Plastic and Reconstructive Surgery	Wound Dressings
	Artificial Skin
Urology	Bulking Agent for Incontinence
Drug Delivery	Cancer Therapeutics; Growth Factors
Orthopedics	Bone Repair

polymyositis/dermatomyositis (PM/DM) has been suggested, but there has never been any statistical proof for these claims (Soo *et al.*, 1993). Another problem with collagen has been with the use of bovine-derived products, since the outbreak of bovine spongiform encephalitis (BSE) in European cows. This has led to closer monitoring of all bovine products.

Recently approved products in the fields of hard tissue repair and relief of urinary incontinence lends support for the collagen renaissance. The bovine collagen product, which was developed under the name 'artificial skin', is now known as Integra Artificial Skin and commercialised (Integra Life Sciences, Plainsboro, NJ, USA). Another product, originally named 'living skin equivalent', made from living-cell-containing bovine collagen is commercially available as Apligraf (Organogenesis Inc., Canton, MA, USA). Similar collagen type I material derived from equine (instead of bovine) Achille's tendons (Collantamp-E-Fascie, Immocoll GmbH) has recently been approved in the European Community.

The above mentioned commercially available collagen products all utilise collagen's physical properties, rather than its biological properties. As stated in section 1.3.3, collagen and collagen-derived peptides have been shown to be involved in cell attachment, proliferation, haemostasis and chemotaxis. To date, no commercially available collagen products utilising these biological functions have become commercially available. Reasons for this will be discussed later in this work; Chapter 6.

1.5 CHEMOTAXIS

Cell migration is a key aspect of many normal and abnormal biological processes, including embryonic development, defence against infections, wound healing and tumour cell metastasis (Martin 1997). Animal cells move by crawling and the active force is derived primarily from the co-ordinated assembly and disassembly of actin filaments (Nobes & Hall 1999).

Chemotaxis, first described by Pfeffer (1884) is the directional locomotion of organisms or cells in relation to sources of chemical attractants or repellants.

During chemotaxis, cells respond to a chemical factor (chemoattractant) and move in the direction of an increasing concentration of that factor. There is a multitude of chemotactic factors (chemoattractants) and there is considerable specificity in the ability of cells to respond to a given attractant (Wilkinson & Haston 1988). The presence of the appropriate cell surface receptor is required (usually) for a specific cellular response to a particular chemical factor (Martin *et al.*, 1983). Owing to this requirement, most chemotactic factors recruit a single class of cells, such as inflammatory cells, connective tissue cells, or endothelial cells. Distinct chemoattractants arise in a wound at different times and these co-ordinate the influx of cells and blood vessels (Fig. 1.3). A lack of chemotactic factors for connective tissue cells may impair the rate of repair, whereas overproduction of a chemoattractant could result in excessive repair or scarring. Chemoattractants show an enormous range in their effective dose *in vitro*, suggesting different functions *in vivo*.

Although it has not been shown in all systems, the first event in chemotaxis is detection of the chemoattractant by specific cell surface receptors (Schiffman 1979). Cells lacking such receptors are unresponsive. Responding cells are able to detect nanogram quantities of certain attractants, indicating that high affinity receptors on

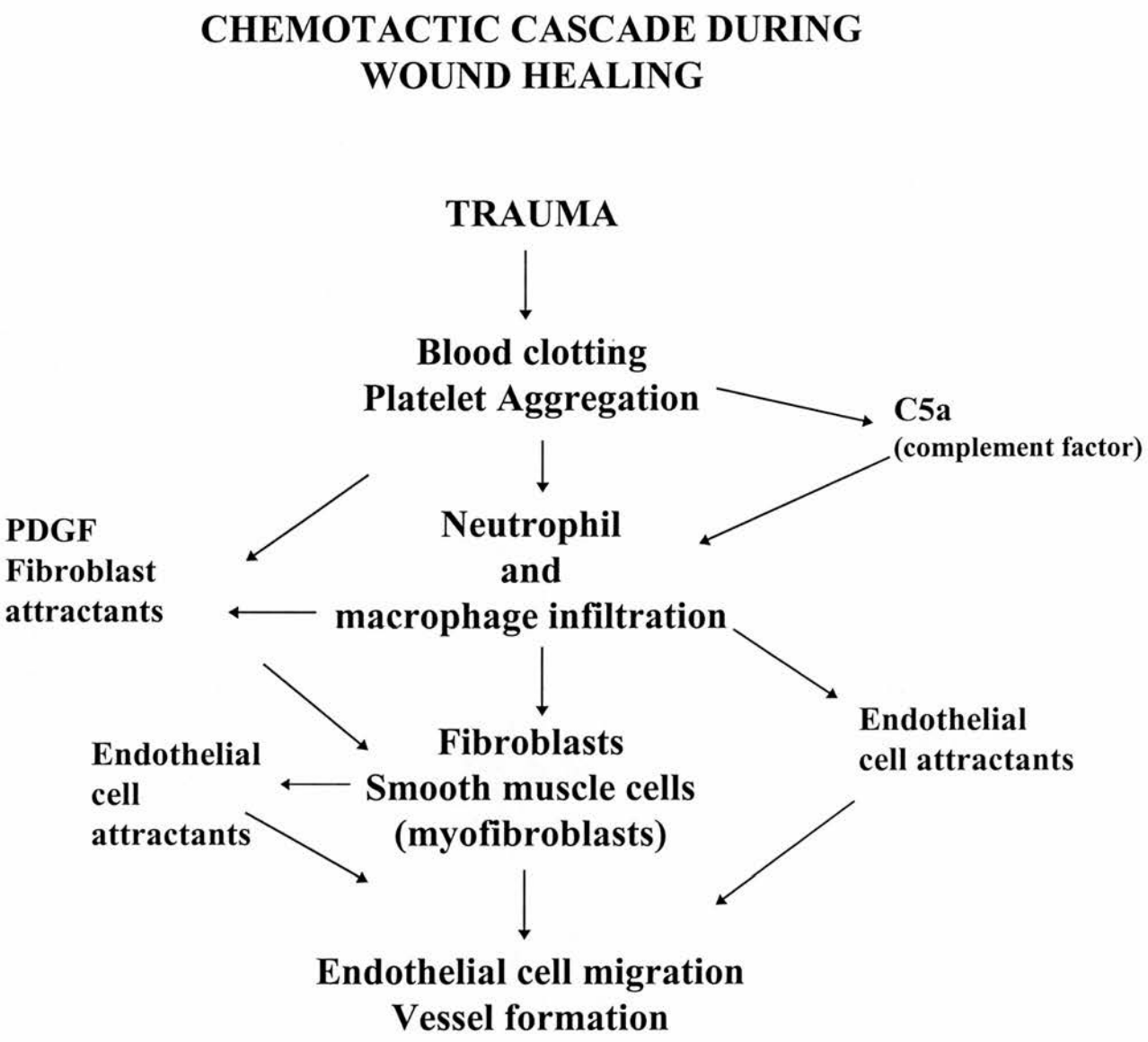

Figure 1.3: Diagram of the cascade of chemotactic factors produced during wound repair (Grotendorst, 1984)

the cell surface are coupled to mechanisms that amplify the signal and activate the cells' motility. Both spatial and temporal mechanisms have been proposed in the detection of the concentration gradient of chemoattractant and the establishment of the direction of movement (Lackie 1986).

It is known that the interaction of an attractant with a cell receptor sets off diverse reactions and that these reactions occur even without a concentration gradient (Raif-Preminger *et al.*, 1995). Thus, it is unlikely that the intracellular reactions required for directed motion differ from those required for random motion. In the case of a chemoattractant, a gradient across the cell body would be expected to cause a differential receptor occupancy along the cell surface. The degree of receptor occupancy is proportional to the magnitude of the chemical reaction it triggers, and the rate of these reactions are greatest in those portions of the cell exposed to the highest concentrations of attractant. This gives rise to an internal gradient in the cell whose polarity could establish direction and whose magnitude could establish the rate of cell movement (Raif-Preminger *et al.*, 1995).

Chemoattractants once bound to the surface receptor stimulate a pleiotropic response in target cells. Specifically, all chemoattractants stimulate both sodium and calcium ion fluxes and changes in lipid metabolism, such as phosphoinositol metabolism and phosphorylation of many membrane proteins as well as cytoplasmic proteins (Downey 1994). These events occur within seconds to minutes after the binding of the attractant by the cell surface receptor. Without the appropriate receptor, no attractant signal can be initiated. Biochemical evidence supports the concept that the biochemical events that regulate cell motility are very similar within a variety of cell types, including endothelial cells, fibroblasts, smooth muscle cells, and even leukocytes. Therefore, whether a fibroblast or an endothelial cell is responding to its attractant, virtually the same internal biochemical mechanisms are at work (Caterina & Devreotes 1991).

The accumulation of cells in a particular site does not constitute evidence for a chemotactic response, and separating the effects of a gradient of chemokinetic factor (affecting random movement) from a chemotactic response (affecting directional movement) is important (Wilkinson *et al.*, 1998). An assay for chemotaxis which has been widely used is the Boyden chamber, in which two compartments are separated by a micropore filter (see section 2.6.1). A method for distinguishing the chemokinetic and chemotactic effects of a substance, the so-called Checkerboard assay (Zigmond & Hirsch 1973), is also widely used.

The injury to connective tissue by immunological, mechanical, physical or chemical injury is followed by an orderly process wherein fibroblasts migrate into the area from surrounding locations, expand their numbers by proliferation and then synthesise and remodel new matrix to constitute scar tissue (Cohen 1979). The mechanisms involved in fibroblast migration are not completely understood, but it is known that as they migrate, they send out lamellipodia which adhere to the substrate (Harris & Dunn 1972). The body of the fibroblast is believed to be drawn up to the new adhesion site by activation of a system of contractile filaments within the cell (Abercrombie *et al.*, 1972). Fibroblasts were first shown by Braum (1971) to migrate *in vivo* and migrate at the site of tissue injury. In a given inflammatory reaction a number of different chemoattractants may be available to recruit new neighbouring fibroblasts to the site (Wilkinson 1988). Table 1.4 shows examples of known fibroblast chemoattractants. This redundancy of chemoattractants may exist to assure that tissue repair is effected, thereby contributing to the survival of the host.

Table 1.4: Fibroblast Chemoattractants (from Postlethwaite & Kang, 1988)

TYPE	REFERENCE
Lymphocyte-derived chemotactic factor for fibroblasts (LDCF-F)	Postlethwaite *et al.*, 1976
Serum (C5)-derived chemotactic factor for fibroblasts	Postlethwaite *et al.*, 1979
Collagen types I, II and III, α chains, and hydroxyproline peptides	Postlethwaite *et al.*, 1978
Fibronectin	Seppa *et al.*, 1981
Tropoelastin and elastin peptides	Senior *et al.*, 1982
Platelet-derived growth factor (PDGF)	Seppa *et al.*, 1982
Leukotriene B_4	Mensing & Czarnetski 1984
PDGF-like factor from SV40/NIH/3T3 cells	Bleiberg *et al.*, 1985
Breast carcinoma cell line-derived factor	Gleiber & Schiffman 1984

1.6 INTEGRINS

Recent research indicates that the major role of the ECM is in the regulation of cell/cell communication rather than passive support of cells (MacNeil 1994). Specific attachment sequences on ECM proteins are recognised by cell surface receptors called integrins (Ruoslahti & Pierschbacher 1987; Ruoslahti 1991; Yamada & Geiger 1997), which are thought to maintain integral cell contact through a bridge between the extracellular structural protein matrix and the cell's internal cytoskeleton. Integrin-dependent cell adhesion regulates not only cell structure and morphology, but also proliferation, migration and differentiation (Cheresh 1991). Integrins are not only biologically important, their medical importance is beginning to be appreciated as well. Integrins have been found to play a role in platelet aggregation, immune functions, tissue repair and tissue invasion (Ruoslahti *et al.*, 1994). The adhesion of cells to each other and to extracellular matrices is a pre-requisite for many cellular functions. Since integrins are cell adhesin receptors, they play fundamental roles in these adhesive events. Integrins can either allow or prevent adhesion. Therefore, they can discourage inflammation and encourage wound healing (Green *et al.*, 1998).

Integrins are a family of transmembrane glycoproteins in which each molecule is a dimer consisting of noncovalently associated α (120–180KDa) and β (90-110KDa) subunits, encoded by separate genes. With 16 α units and 8 β units, there are at least 22 possible integrins (Hemler 1999). It is thought that the particular integrins which a cell expresses are largely responsible for determining whether and how that cell will interact with the ECM. Some cultured mammalian cell lines express only a few integrins whereas others can have as many as 10 (Vogel *et al.*, 1990). Signalling information originating from binding interactions between integrins and ECM molecules (ligands) is transduced across the plasma membrane into a variety of signal transduction pathways in a process termed "outside-in signalling" However, intracellular regulators modify external integrin ligand binding properties in a process termed "inside-out" signalling (Yamada 1997). If the integrin is not expressed, the cell

will not attach to ECM proteins. A low level of expression may allow light attachment and the possibility to migrate along the protein. High level expression may result in the cell being anchored in place to the ECM. ECM proteins act as chemical messengers by interacting with the cell surface through specific receptors i.e. the ECM can stimulate chemical messages from which cell behaviour can be altered (Meredith *et al.*, 1993). Tyrosine-phosphorylated proteins have recently been detected in a range of cells following integrin ligand binding, (Juliano & Varner 1993). An increase in cytoplasmic pH has also been detected, and integrin activation can lead to gene expression for cytokines and metalloproteinases (Ingber 1991). The specificity and affinity of a given integrin receptor on a given cell are not always constant. Both activation and deactivation of integrin functions have been reported. There is increasing evidence that integrins do mediate information transfer into cells (Juliano & Haskill 1993).

Most integrins are expressed on a wide variety of cells, and most cells express several integrins. Individual integrins can often bind to more than one ligand. Equally, individual ligands may be recognised by more than one integrin. Integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$ are the major integrin collagen receptors (Knight 2000). The $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins are known to bind collagen and laminin with the sequence Asp-Gly-Glu-Ala (DGEA) often being recognised (Vandenberg *et al.*, 1991).

The recognition site for many of the integrins that bind to extracellular matrix and platelet adhesion proteins is the tri-peptide arginine-glycine-aspartic acid (RGD) (Ruoslahti & Pierschbacher 1987). There are many RGD sequences in collagens, but judging from lack of inhibition by RGD-containing peptides of the integrin binding to collagen in many cell types and integrin-binding activity in collagen fragments that do not contain any RGD sequence (Gullberg *et al.*, 1992), at least most of the collagen-mediated cell attachment is not RGD dependent.

Integrins that recognise collagen can modulate cell behaviour, including adhesion and spreading, migration, division, metabolism and the expression of the differentiated phenotype. These important processes are physiologically relevant to growth and development, wound repair and angiogenesis and in pathological processes such as thrombosis and tumour metastasis (Knight 1998).

It has been demonstrated that the integrin $\alpha 2\beta 1$ mediates the Mg^{2+}-dependent adhesion of platelets to collagen (Santoro *et al.*, 1986). Adhesion by the $\alpha 2\beta 1$ receptor is specific for collagen. Collagen types I, II, III, IV and VI support adhesion via this receptor while other adhesive proteins do not (Staatz *et al.*, 1989). The $\alpha 2\beta 1$ integrin serves as a cell surface collagen receptor on platelets, fibroblasts, endothelial and some epithelial cells. The binding site for this integrin has been shown to be contained within the $\alpha 1$(I) CB3 fragment of collagen (Staatz *et al.*, 1990). As previously mentioned, this fragment is involved in platelet adhesion. The amino acid sequence Asp-Gly-Glu-Ala (DGEA) is an important determinant of the $\alpha 2\beta 1$ recognition site (Staatz *et al.*, 1991). Also in Type IV collagen, a dominant $\alpha 1$ integrin binding site is located in the CNBr fragment CB3 (Vandenberg *et al.*, 1991).

1.7 AIM

Peptides derived from collagen, elastin, fibronectin and laminin display chemotactic activities towards neutrophils, macrophages, fibroblasts, endothelial and epithelial cells. These breakdown products have been shown to a play a role in the healing process. However, the characterisation of active peptides has not been performed previously in a systematic manner and comparison of potencies is difficult. Therefore, the aim of this study was to isolate and identify naturally occurring peptide sequences from collagen which have biological activities. The target responses chosen were chemotaxis, proliferation and cellular attachment to a substrate in populations of fibroblasts and endothelial cells. Since these cells are of critical importance in tissue repair, a further aim of the study was to assess the potential for the development of small collagen-derived peptides as a therapeutic treatment for impaired wound healing. The implications being that the peptide(s) could eventually then be used *in vivo* to enhance the wound healing process.

CHAPTER 2:

MATERIALS & METHODS

2.1 PREPARATION OF COLLAGEN

A mixture of collagen types I and III was extracted from the skin of stillborn pigs (see below). Type V collagen; from pig placenta was given as a kind gift from Dr. Gordon Paul (Bristol).

2.1.1 Pepsin extraction of collagen

The method used for the pepsinisation of collagen was based on that of Miller and Rhodes (1982). Pepsinisation aids in the separation of Type I and Type III collagens.

Pig skin, containing collagen (60g), was digested with pepsin (6g; Sigma, Poole, Dorset) in a ratio of (collagen:pepsin) 10:1 (w/w), in 1 litre of 0.5M acetic acid at 4°C for 24hrs (Figure 2.1). This method relies on the cleavage of the cross-linked non-helical terminal peptide region of polymeric collagen and the resistance of the collagen triple helix to enzymatic degradation below its denaturation temperature. The collagen triple helix is unique in being both resistant to pepsin and soluble at acid pH, so that the end product of digestion is a mixture of solubilised collagen types.

2.1.2 Salt Precipitation

Salt fractionation was adopted to separate the collagen types (Figure 2.1). This method is based on the principle that various collagen types have different solubility properties at different salt concentrations.

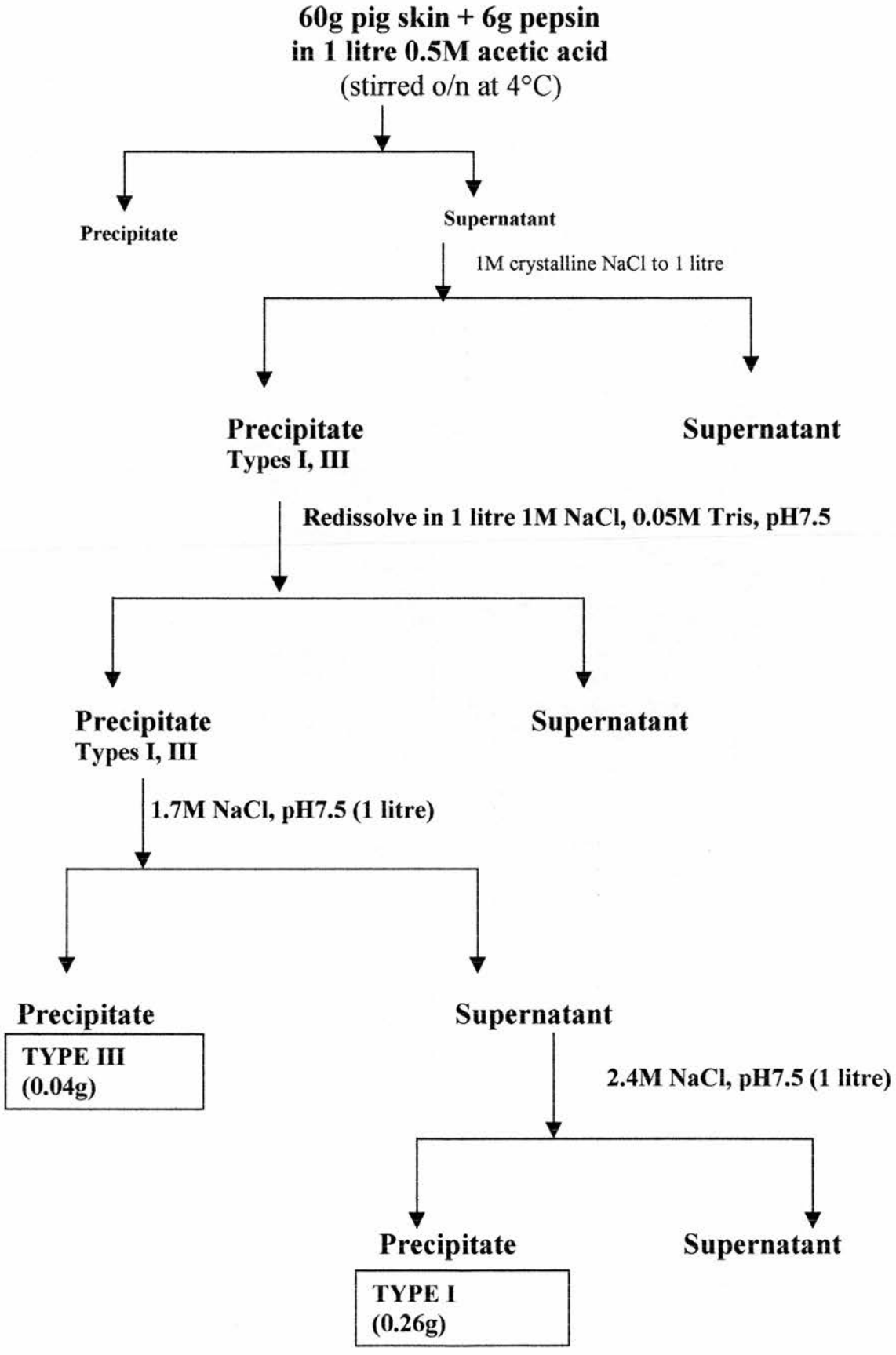

Figure 2.1: Procedure for the isolation of collagens (adapted from Miller & Rhodes, 1982)

All procedures were carried out at 4°C (to minimise proteolytic digestion) and centrifugations were at 30,000g for 1hr. Collagens were then dialysed against H_2O and freeze-dried. Purity and identification of collagens was carried out by SDS-PAGE analysis.

2.2 SODIUM DODECYL SULPHATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

2.2.1 Introduction

SDS-PAGE is used to resolve and separate individual components of protein mixtures, in this case collagen components (chains and peptides).

Discontinuous SDS-PAGE is a widely used technique, which separates proteins according to their molecular weight (Garfin 1990). SDS is a negatively charged detergent that solubilises and binds to proteins such that approximately 0.5 mol of SDS is bound per amino acid residue. This large amount of anionic detergent is sufficient to overwhelm the intrinsic charge on the polypeptide chain so that the net charge per unit mass becomes approximately constant. In the presence of SDS and the reducing agent β-mercaptoethanol (which reduces disulphide bonds) proteins are denatured and adopt an extended coil configuration. Electrophoretic migration is then determined by the effective molecular length or approximate weight of the polypeptide chain. Molecular weights can be estimated from a plot of distance migrated versus log molecular weight, using proteins of known molecular weights as standards.

Polyacrylamide gels result from the polymerisation of acrylamide monomer into long chains and the crosslinking of these chains by bifunctional compounds such as N,N'-methylene bisacrylamide (BIS). Ammonium persulphate initiates this polymerisation, with N,N,N',N'-tetramethylethylenediamine (TEMED) catalysing the formation of free radicals from the persulphate. In the gel media, the passage of any particle is hindered by the structure of the matrix, and the extent depends upon the relative sizes of the particles and pores in the gel network. The pore

size is altered by changing the acrylamide concentration.

2.2.2 Method

Discontinuous SDS-PAGE was performed in vertical slab gels using the buffer system of Laemmli (1970). Gels containing 4.5% (stacking gel), 6% or 12% (separating gel) were prepared from a stock solution of 30% (w/v) of acrylamide and 0.8% (w/v) of N,N'- methylene bisacrylamide. The final concentrations in the separating gel were as follows: 0.375M Tris-HCl (pH 8.8) and 0.2% (w/v) SDS. The gels were polymerised chemically by the addition of tetramethylethlenediamine (TEMED; 25µl/75ml separating gel) and ammonium persulphate 2% w/v (1.5ml/75ml separating gel). Gels 13cm x 14cm were prepared in 16cm x 18cm glass plates with 1.5 mm spacers. The stacking gels of height 2cm contained 0.125M Tris-HCl (pH6.8) and 0.2% SDS (w/v) and were polymerised chemically by the addition of TEMED (25µl/25ml stacking gel) and 2% (w/v) ammonium persulphate (0.625ml/25ml stacking gel). The electrode buffer (pH 8.3) contained 0.025M Tris, 0.192 M glycine and 0.25% SDS (w/v). The samples contained the final concentrations: 0.1M Tris-HCl (pH 6.8), 2% (w/v) SDS, 10% (v/v) glycerol, 3% (v/v) β-mercaptoethanol and 0.001% bromophenol blue as tracking dye. The proteins (1mg collagen/ml) were completely denatured by immersing the samples, in microcentrifuge tubes, for 3 minutes in boiling water. The samples (100µl) were then loaded into the wells using a 100µl microsyringe (Hamilton Bonaduz AG., Bonaduz, Switzerland). Electrophoresis was carried out at 40mA constant current per gel until the bromophenol blue marker reached the bottom of the gel (about 3½ hrs). Gels were fixed and stained simultaneously overnight in 250ml 8% (v/v) acetic acid/50% (v/v) methanol containing 0.1% (w/v) Coomassie

Brilliant Blue R-250. Gels were then destained 5-6 hrs by continuous shaking in several changes of 8% (v/v) acetic acid. Gels were destained until background staining levels were minimal.

2.3 DEGRADATION OF COLLAGEN

2.3.1 Cleavage of peptides with CNBr

The action of CNBr upon proteins is unique in its selective attack on methionine (Gross 1967). When a methionine residue reacts with CNBr it is converted to a residue of homoserine lactone, which under acidic conditions is in equilibrium with a residue of homoserine. The formation of homoserine or its lactone results in the liberation of the amino group of the residue that followed methionine. Accordingly, a residue of methionine which originally occupied an endo-position is converted to homoserine (lactone), which becomes the C-terminal residue of a peptide fragment. The reaction takes place under acidic conditions in order to denature the protein and to expose the side chains of methionine to attack by CNBr, which may be added in excess.

Samples(2mg) were dissolved in 1ml of 70% Formic acid and digested with CNBr (2mg CNBr/1mg protein) at 25°C (Seyer 1977) overnight. After digestion, samples were diluted 10 fold with cold H_2O and freeze-dried.

2.3.2 Fractionation of CNBr peptides

Type I collagen CNBr peptides were fractionated by cation-exchange FPLC (Bateman *et al.*, 1986). Freeze-dried CNBr peptides (0.5mg) were dissolved in 1ml of starting buffer (0.02M sodium formate, pH 3.8, containing 0.07M NaCl and 2M urea) and denatured at 50°C for 3 minutes. The samples were filtered using 0.22μm filters (Sartorius). The filtered samples (0.5mg/ml) were chromatographed at room temperature

on a Mono S HR 5/5 cation-exchanger (Pharmacia). Following injection of the sample, the starting buffer was delivered for 5 minutes at a flow rate of 1ml/min. A 65-ml linear gradient was then commenced at the same flow rate. The gradient was from 0.07 to 0.26M NaCl in 0.02M sodium formate, pH 3.8, containing 2M urea. Fractions containing the separated peaks were collected, dialysed against 0.1M acetic acid and concentrated by freeze-drying. The identities of the separated CNBr peptides were confirmed by SDS-PAGE (12% acrylamide) as by method of Bateman *et al.*, 1986).

2.3.3 Quantification of peptides obtained from collagen by treatment with cyanogen bromide

Collagen quantitation was based on the Sircol Collagen Assay (Qubis Ltd., Belfast). The assay is based on the specific binding by collagen of a dye reagent that is free from interference from non-collagenous proteins (Anderson & Elliot 1991). The assay is rapid, sensitive and easy to use. The assay kit comprises components: the Sircol dye reagent, an alkali reagent, a collagen standard and a salt-soluble reagent that precipitates collagen.

The collagen standard (prepared from a 1mg/ml stock solution of collagen I in 0.5M acetic acid), and reagent blanks were used to produce a straight-line calibration curve with a Dynatech automated spectrophotometer at 540nm. The intensity of the colour at 540nm was found to be proportional to the collagen concentration in the sample. The collagen standard was run in duplicate at three concentrations, using 12.5, 25 and 50µl aliquots.

To the test sample (50µl of purified peptide in 50µl of 0.5M acetic acid) 1ml of Sircol dye reagent was added. After mixing at room temperature for 30 mins, the tubes were then centrifuged at 10,000g for 5 mins. The supernatant, containing unbound dye, was then discarded. The collagen-bound dye remained as a pellet at the bottom of the tube. To the collagen-dye complex 1ml of alkali reagent (0.5M sodium hydroxide) was added. This was vortexed to bring the bound dye into solution. The dye reagent selectively precipitates the collagen molecules from mixed protein populations.

The absorbances of assay blanks, standards and collagen peptides were measured at 540nm. The reagent blank value was subtracted from all readings (standards and unknowns). A standard curve was plotted from which the collagen concentrations of unknown samples were obtained by interpolation.

2.3.4 Digestion by Bacterial Collagenase

Samples (10mg collagen) were dissolved in 10mls of 50mM Tris-HCl pH 7.5 containing 0.15M NaCl, 5mM $CaCl_2$, 10mM NEM and incubated for 24hrs at 37°C with bacterial collagenase at a ratio of enzyme:collagen 1:100 (w/w). The reaction was stopped by heating to 90°C for 4 hrs (Levitsky *et al.*, 1994).

2.3.5 Sequential Enzymatic Digestion

In order to create smaller peptides, the collagen was degraded with two enzymes, sequentially. After collagenase treatment, further breakdown was carried out by trypsin in one case, and with chymotrypsin in another.

These enzymes can only degrade collagen after the triple helix has been broken up by collagenase.

For the double enzymatic digestions, collagen (10mg) was digested with collagenase (100μg) in 10mls of buffer as described above. The collagenase (Sigma) was inactivated as before. A second digestion was carried out by the addition of trypsin or chymotrypsin (100μg/ml).

The new enzyme treatment was also carried out for 24hrs at 37°C. Again the reaction was stopped by heating to 90°C for 4hrs.

2.4 REVERSE PHASE HPLC

2.4.1. Introduction

Reverse-phase HPLC (High Performance Liquid Chromatography) has proven to be an invaluable tool for the analysis and purification of proteins and peptides. It is very effective in separating peptide fragments from enzymatic digests because of its high resolving power. It is able to resolve very similar polypeptides, some of which differ by a single amino acid (Willard *et al.*, 1988).

Polypeptides are large molecules and cannot "partition" into the stationary-phase, as do small molecules. Polypeptides are adsorbed onto the hydrophobic -"reverse phase"- surface from the mobile phase and remain adsorbed until the organic component of the mobile phase reaches a critical concentration, at which point the polypeptide is desorbed and carried by the mobile phase to the column exit with little further interaction with the reverse phase surface. Polypeptides are eluted quickly once the critical organic concentration is reached, which accounts for the sharp peaks and high resolution.

2.4.2 Method

Digested collagen peptides were resolved using a C18 reverse phase column (Dynamax 300A, 25cm long, 4.7mm ID). Acetonitrile was obtained from Rathburn Chemicals Limited, Scotland and trifluoroacetic acid from BDH Ltd, Poole, England. All solvents and buffers were membrane filtered (0.22μm, Sartorius).

Freeze-dried collagen samples (1mg) were dissolved in 1ml of 0.1% (v/v) trifluoroacetic acid. Samples were filtered using 0.22μm HPLC filters. Filtered samples were injected and chromatographed at room temperature on a C18 column that was equilibrated with 0.1% (v/v) TFA. A 120ml gradient of 0-40% acetonitrile was delivered at a flow rate of 1ml/min. The fractions were collected at 10 min intervals and freeze-dried individually.

2.5 CELL CULTURE

2.5.1 Cell types used

The cells used in the experiments were:

a) L929 mouse connective tissue fibroblasts (Sandford *et al.*, 1948; kindly supplied by Professor M. Ferguson, Department of Cell and Structural Biology, Manchester University).

b) Bovine Aorta Endothelial Cells (Booyse *et al.*, 1975; BAEC, kindly supplied by Dr. S.Srivastava, Bioengineering Department, University of Strathclyde).

c) Rat Wound Fibroblasts (RWF) - see Materials & Methods 2.5.3.2 (Finesmith *et al.*, 1990).

d) 3T3 mouse embryo fibroblasts (Todaro & Green 1962; also supplied by Prof M Ferguson).

BAEC are a primary cell line and after a large number of passages they become senescent. Therefore, cells used in the experiments were between 15 and 30 passages. RWF also senesce and were therefore only used up until 10 passages. Different cell lines senesce at different rates, hence the RWF and BAEC cell lines were used at different passage numbers. L929 cells are from an established cell line i.e. they have the potential to be subcultured indefinitely *in vitro*, therefore the passage number of these cell types is not thought to be important.

Fibroblasts and endothelial cells types were studied as both are essential to wound healing (Clark 1996). It was also important to examine for differences between cell lines, hence three types of fibroblast cell lines were chosen in these studies.

The cells were grown in 25cm^2 cell culture flasks and incubated at 37°C in a high humidity atmosphere containing 5% CO_2/95% air.

2.5.2 Cell Culture Reagents

All cell types were maintained in Dulbecco's modification of Eagle's medium (DMEM) containing 10% foetal calf serum (FCS), 1% penicillin/streptomycin solution, 1% glutamine and 9mM sodium bicarbonate (GIBCO/BRL). The amount of FCS in the formula was adjusted to 0% or 2% for the proliferation experiments. The medium was stored at 4°C and used within one month of the preparation date.

Hank's balanced salt solution (HBSS), without calcium and magnesium (GIBCO/BRL) was used to rinse cell monolayers before use. This is done to remove traces of the serum which would inactivate the action of trypsin, used to detach cell monolayers. Trypsin solution was prepared by ten-fold dilution of 10X concentration of trypsin-EDTA (GIBCO/BRL) with HBSS.

2.5.3 Isolation of Cells

2.5.3.1 Rat Wound Fibroblasts

These cells were isolated following the method of Finesmith *et al.*, (1990.) Adult Wistar rats were implanted with four polyvinyl alcohol sponge discs (1cm diam.) beneath the panniculus carnosus, next to the body musculature. The sponges were removed after 10 days and placed in 20 mls DMEM containing 20% FCS and amphotericin (25µg/ml), streptomycin (1,000 µg/ml) and penicillin (1,000 U/ml) and placed on ice.

Under sterile conditions encapsulated adventitia were removed, and the sponges were minced and placed in DMEM with 10% FCS, 200 units of bacterial collagenase (Type 1A, Sigma), penicillin and streptomycin in the above concentrations, and amphotericin (2.5ug/ml) at 37°C, in a 5% CO_2 atmosphere. After 4 hrs, the minced sponges were agitated by pipetting, and the resulting supernatants were centrifuged at 1,200 rpm, 25°C for 7 minutes. The pellets were resuspended in DMEM with 20% FCS and antibiotics (penicillin, 100U/ml; streptomycin, 100µg/ml; amphotericin, 2.5µg/ml) and placed in 75cm^2 flasks. After the first passage the cells were grown in 10 mls DMEM with 10% FCS and antibiotics in the above concentrations, with the omission of amphotericin.

2.5.4 Microwell Plate Cultures

Medium was aspirated from the confluent cell monolayers and replaced by 5ml of HBSS added 5 minutes before removal. Trypsin solution (1ml) was then added to the flask, which was incubated until all the cells had rounded and detached from the flask surface. DMEM-10% FCS (20mls) was added to the cell suspension to inhibit the trypsin. Cells were centrifuged at 1,200 rpm for 5 minutes and washed twice in DMEM. A haemocytometer (improved Neubauer) was used to count the number of cells per millilitre, then the cell suspension was diluted with DMEM to give the desired concentration (5,000 cells/well). 100µl volumes of cell suspension were added to a 96 well plate (Costar UK Ltd) using a multichannel pipette with sterile tips. 96 well plates are used for ease of handling and economy of scale. Cell proliferation or attachment assays were then carried out, as described below.

2.5.5 Effect of collagen peptide on cell proliferation

To test the effects of collagen peptides on the proliferation of endothelial cells and fibroblasts, the cells were prepared as before (section 2.5.2), then suspended in DMEM containing 2% FCS and counted using a haemocytometer. Cells were seeded at 5,000 cells per 96-well plate and allowed to attach overnight to the tissue culture plastic. The collagen fragments were then tested for their effect on cell proliferation over a range of concentrations (1pg/ml-100µg/ml).

Collagen peptides, dissolved in DMEM containing 2% FCS and filter sterilised, were incubated with adherent cells for 1, 2 and 5 days. Cell number was then assessed using the Methylene Blue Assay (section 2.5.7) and cell growth in the presence of collagen peptides expressed as a percentage of cell growth in control cultures containing 2% DMEM. Other controls used were DMEM containing no FCS (0%) as a negative control and DMEM containing 10% FCS as a positive control. The cells used in these experiments are grown in DMEM. These cells grow best when the media is supplemented with 10% serum. This has been considered the positive control in many experiments. Less serum is needed in order to slow growth e.g. 2%. Cells grown with DMEM only (0% serum added) have been used as negative controls, as cells grow extremely slowly without the addition of serum. Cells grown in 2% serum give optimal conditions for monitoring the effects of collagen peptides on growth.

2.5.6 Effect of collagen peptide on cell attachment

Cells were prepared as for the proliferation assay. Here both collagen peptide preparations, dissolved in DMEM containing 2% FCS, plus cells were added to the plates at the same time. The cells were then allowed to attach to the tissue culture plastic for 0, 30, 60, 90, 120, 180 and 240 minutes. Attachment was then determined again using the Methylene Blue Assay and attachment in the presence of collagen peptides was assessed by comparison to control cells grown only in the presence of 2% DMEM.

2.5.7 Methylene Blue Assay

This is a method described by Oliver *et al.*, 1989. At pH 8.4 methylene blue is a basic dye that is positively charged. It binds to anionic groups in fixed cell monolayers i.e. phosphate in DNA and RNA, and phosphorylated proteins. Hence the amount of dye binding varies from one cell type to another. The addition of HCl lowers the pH to below pH 2 causing protonation of the acidic groups and liberation of the dye into the elution solvent. Methylene Blue solution does not obey Beer's Law in aqueous solution at low pH due to the formation of dimers. When ethanol is added to the HCl, dimer formation is suppressed and Beer's Law is obeyed.

Cell layers were fixed in a 96 well microwell plate with 100µl methanol for 5 minutes. These were then stained with 100µl Methylene Blue (0.1% (w/v) in 10mM borate buffer, pH 8.4) for 30 mins. The dye was rinsed until clear with 400µl of 10mM borate buffer three times. Methylene Blue was eluted from cell layers with 100µl of 0.1M HCl containing 20% ethanol. The absorbance at 630nm was measured using a Dynatech automated multichannel spectrophotometer. The mean, standard

deviation and standard error for each sample (n=6) were calculated. To test for statistical significance t-Test analysis was carried out comparing each sample with the DMEM+2%FCS control. Six samples (n=6) were required to obtain minimum standard deviation.

2.6 CHEMOTAXIS

2.6.1 Boyden Chamber Assay (*in vitro* assay)

Chemotaxis of cells through Millipore filters was quantified by the modified Boyden Chamber technique (Falk *et al.*, 1980) using a Neuroprobe 48-well microchemotaxis chamber. The original membrane technique was first described by Boyden in 1962. This is a specially designed chamber consisting of two compartments separated by a porous filter (Figure 2.2). The pore size used varies depending on the cell type examined as follows:

neutrophils-3μm pores

monocytes/macrophage-5μm

fibroblasts, endothelial cells-8μm

Cells were prepared as before (see section 2.5.4), 50μl of 4 x 10^5 cells/ml in DMEM, and placed in the top compartment. The putative attractant (27μl) was added to the lower compartment.

Samples being tested must contain at least 50% serum-free maintenance medium (DMEM), as the samples should be in the same solution as the cells. Glycylglycine-buffered saline (GGBS; 0.015M glycylglycine/0.14M NaCl) pH 7.2 was used to dilute and suspend test samples (fibroblasts migrate better in this compared to other saline solutions; Postlethwaite & Kang 1988). Therefore each sample was prepared in 50% GGBS + 50% DMEM. 27μl of each test sample was added to the Boyden Chamber wells. A polyvinylpyrrolidone (PVP)-free polycarbonate filter (Co-Star) was placed over the samples. A rubber gasket was put over the filter and the top chamber attached. The chamber was then incubated for 10 mins at

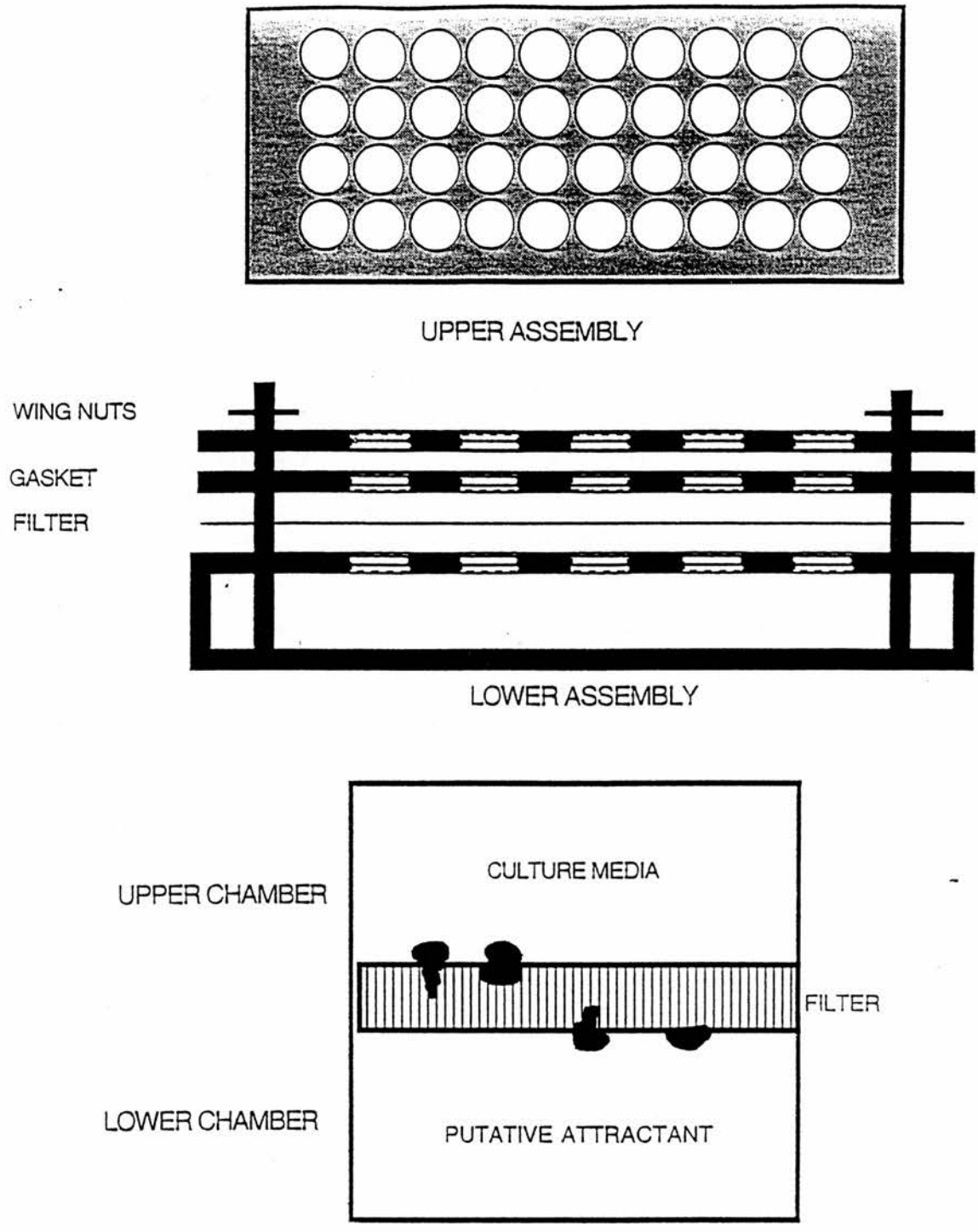

Figure 2.2: Boyden Chemotaxis Chamber

Cells are introduced into the upper chamber. Cells respond to a concentration gradient when a putative attractant placed in the lower chamber diffuses through the filter into the upper chamber. Cells reaching the bottom surface of the membrane are fixed, stained and counted.

37°C to reach temperature equilibrium. The fibroblasts were then added to the top chamber (50µl) and incubated for 2.5 hours in a CO_2 incubator. The filter was then removed and dipped in H_2O to remove non-attached cells. Attached cells were fixed in methanol and stained with Diff-Quick, a modified Giemsa stain. The membrane was then dried on a glass slide. Cells were counted at x40 magnification. Twenty fields of view were required to give an accurate mean value for the number of cells counted.

All samples were tested in triplicate and this was repeated three times on separate occasions. The SEM was calculated for each sample. DMEM+GGBS was used as a negative control to account for random cellular migration.

2.6.2 Checkerboard assay

Most chemoattractants can increase both directed (chemotaxis) and random (chemokenesis) motility of responding cells, but these can be distinguished by using the Checkerboard assay of Zigmond and Hirsch (1973). In this assay cells are set as described for the Boyden Chamber Assay. The cells are exposed to:

1) A range of attractant concentrations but no gradient (this provides a dose-response curve for non-chemotactic effects of the attractant).
2) A range of positive gradients
3) A range of negative gradients.

If the factor has a chemotactic effect, cells would be expected to penetrate deeper into the filter in 2 than in 1, and less far into the filter in 3 than in 1. Here the concentration gradient of the putative attractant is

varied in the upper and lower compartments of the chamber. Chemicals that stimulate migration equally when placed in an equal concentration below and above the filter are characterised as affecting random motility. Attractants that are much more potent when placed below the filter are judged to elicit true directed motility.

In the top wells of the chamber, various concentrations of the collagen peptides (100ng/ml-10μg/ml) were applied together with fibroblasts at a concentration of 4×10^5/ml (total volume 50μl). In the bottom wells, the same concentrations of the peptides (27μl) were placed such that several possible concentrations above and below the filter were tested. Each combination was tested in triplicate. Filters were stained and cells counted as for the chemotaxis assay.

2.7 PVA SPONGE MODEL FOR CELLULAR INFILTRATION

2.7.1 PVA Sponge Preparation

PVA (polyvinyl alcohol) is used as an inert substance which is porous and allows cells to migrate into it, therefore it can be used to measure cellular infiltration. Sponges were prepared and sterilised prior to implantation into rats. The sponges were cut to a uniform size (1cm diameter) and weighed. Thus the dimensions of the sponges are controlled prior to their implantation and therefore the invasion of granulation tissue is not affected by this parameter. 1cm discs were bored out of a PVA block using an electric drill and cork borer and then saturated with water. These were then dried in an oven and weighed. Sponges in the 14-20mg range were used for implantation. The sponges were washed overnight in tap water, then in deionised water prior to autoclaving. They were then ready to be implanted.

2.7.2 Implantation Method

PVA sponges were implanted subcutaneously into adult male Wistar rats (4 sponges per rat, 2 per side). Five rats were used at each time point for each sample under investigation. Three days after implantation, two sponges per rat were injected with 0.1ml of collagen peptide sample (conc. varying from 100μg/ml-3mg/ml) and two sponges with 0.1ml of PBS control buffer. The rats were killed at days 7 and 10 and the sponges removed.

2.7.3 Homogenisation and preparation of PVA sponges for analysis

The PVA sponges were prepared for biochemical and histological analysis. Immediately after the sponges were removed from the rats the adventitious tissue was removed. The sponges were kept on ice at all times in order to restrict degradation of protein and DNA. The sponges were halved and weighed, one half for histology, the other for biochemical analysis (protein, DNA and collagen). The sponges were homogenised using an Ultraturrax blade followed by centrifugation (3,000rpm, 25°C for 15mins). 2ml of the supernatant was removed for the hydroxyproline assay and the remainder was used for DNA and protein assays.

2.7.4 Histology

Histological analysis of extracted sponges was kindly carried out by Stirling Royal Infirmary. Each sponge was assessed for content of granulation tissue (a loose collection of fibroblasts, inflammatory cells and neovasculature) by examining Haemotoxylin and Eosin (H&E) stained sections of each sponge. Haematoxylin binds to nucleic acids, allowing them to be visualised; the eosin is then used as a counter stain to highlight other components, such as mitochondria and cytoplasmic constituents (Young & Heath 2000). Three sections of each sponge were cut at 50μm intervals over a total distance of 100μm. The granulation tissue in each set of sponges (control against collagen peptide) was assessed microscopically by comparing the content of the sponge containing the peptide to those containing PBS control.

2.7.5 Biochemical Testing

2.7.5.1 Assay for Protein

The method used for determination of protein content in the PVA sponges after their removal from rats was based on that of Bradford (1976). This assay is based on the principle that the binding of the dye to any protein present in the sample causes a shift in the absorption maximum of the dye from 465nm (red form) to 595nm (blue form). Serial dilutions of protein standards (BSA, Sigma) were prepared, from which a standard curve was produced. Protein concentrations in the sponge samples could be calculated from this. 5ml of protein binding dye was added to 50µl of each sample in triplicate, the sample was vortexed and its absorbance read at 595nm.

2.7.5.2 Assay for DNA

The method used to determine the DNA content in the PVA sponges is described by Gendimenco *et al.*, (1988). This method is a modification of the diphenylamine colorimetric assay (Burton 1956). A coloured solution absorbing maximally at 600nm is produced by reacting acid-hydrolysed DNA with phenylamine in the presence of sulphuric acid, acetic acid and acetaldehyde. Serial dilutions of calf thymus DNA (Sigma) were prepared from which a standard curve was produced. 0.5mg of samples were resuspended in 0.5mls of 0.5M perchloric acid and then heated at 90°C for 30mins. Aliquots (50µl) of samples and standards were added in triplicate to 96-well plates, and 100µl of diphenylamine colour reagent was added to each. The plates were sealed and incubated at 37°C overnight. The absorbance at 600nm was measured using a Dynatech automated multichannel spectrophotometer. From these values and the

standard curve the DNA content of PVA sponges could be derived.

2.7.5.3 Assay for Collagen

Hydroxyproline is an amino acid unique to collagen and therefore its estimation gives an accurate estimate of the collagen content in the sample. The hydroxyproline content was determined using HPLC of dansylated amino acids where it was also possible to determine the concentration of proline (Negro *et al.*, 1987). Hydroxyproline concentrations are different for each collagen type e.g. 14.2% in collagen I and 17.23% in collagen III (Miller & Gay 1982).

Sponge samples were hydrolysed in 6M HCl overnight at 105°Cin sealed tubes. Samples were dissolved in 0.25ml lithium borate buffer. Samples were then dried with compressed nitrogen in a fume hood, filtered (0.45μm filter) and dansylated before being loaded onto the HPLC autosampler in preparation for analysis. Hydroxyproline standards were prepared and analysed in order for a standard curve to be formed from which the levels of hydroxyproline and hence collagen, in the sponges could be calculated. The HPLC column used was Spherisorb C18 column ODS, 3μm, (25cm x 4.6mm). The mobile phase used was 86% (25mM acetic acid + 25mM sodium dihydrogen orthophosphate) and 14% acetonitrile. The amino acids were washed from the column with acetonitrile and the derivatised amino acids were detected by UV absorbance at 254nm.

2.8 AMINO ACID SEQUENCING

The isolated active collagen peptide was subjected to automated sequencing on an Applied Biosystems Model 477A Microsequencer with a Model 120A on-line phenylthiohydantoin (PTH) analyser (Hayes *et al.*, 1989). This process was carried out by Dr. A Cronshaw, Edinburgh University. Applied Biosystems chemicals were used throughout this procedure.

2.9 SYNTHETIC PEPTIDE SYNTHESIS

The collagen peptide with the strongest chemotactic properties was synthesised by the Chemistry Department, University of Edinburgh on a fully automated Applied Biosystems 430A peptide synthesiser. The Fmoc/^{t}Bu based method of peptide synthesis was used which involves the use of the base-labile 9-fluorenylmethoxycarbonyl N^{α} protecting group in conjunction with acid-labile side protection and peptide-resin linkage (Carpino & Han 1972). The following protecting groups were used; Hyp(O^{t}Bu), Glu(O^{t}Bu), Arg(Pmc). Gln was incorporated without side chain protection. The peptide was synthesised on a 0.25mM scale using Fmoc-Glycine functionalised 4-alkoxybenzylalcohol (Wang) resin and all amino acids were incorporated using double coupling cycles. Each synthetic cycle involved 1) treatment with acetic anhydride (to cap any free amino groups prior to N^{α} deprotection), 2) Fmoc removal by treatment with the organic base piperidine and 3) coupling of the next amino acid in the sequence. In this way the desired peptide was built up from the C to N terminus. The peptide was then cleaved from the resin with simultaneous removal of side chain protecting groups by treatment

with a mixture of TFA/ethanedithiol/triisopropylsilane/thioanisole and water for 3 hours at room temperature. The resin was then removed by filtration, the TFA evaporated and the peptide isolated by precipitation with diethyl ether and filtration. The crude peptide was then purified by reverse phase HPLC and lyophilised. Laser Desorption Mass Spectrum and analytical HPLC were carried out on the pure peptide.

2.10 STATISTICAL SIGNIFICANCE

Statistical significance was set at a p value of less then 0.05, and described as highly significant at a p value of less then 0.01.

CHAPTER 3:

BIOLOGICAL EFFECTS OF COLLAGEN I AND III CNBr PEPTIDES

3.1 INTRODUCTION

Collagen is a protein present throughout the animal world and is the main constituent of skin. Cell-collagen interactions are essential to cell movements in inflammation, wound healing, trophoblast implantation, fetal development and cancer (Minafra *et al.*, 1985). Collagen Types I, II and III and their derived-peptides have been shown to be chemotactic towards a variety of cell types (see Chapter 1.3.3.3).

Cyanogen bromide (CNBr) cleavage of collagen chains and tryptic cleavage of individual CNBr peptides was used to prepare fragments for structural microanalysis. The action of CNBr upon proteins is unique in its selective attack on methionine residues (Gross 1967).

Cyanogen-bromide-derived peptides from collagen have been shown to have biological activities *in vitro*. An example of this is their effects on the attachment and migration of neural crest cells (Perris *et al.*, 1993). In addition, peptides CB3, CB6, CB7, CB8 from the collagen α1(I) chain and CB3 and CB4 from the α1 chain of collagen III support platelet adhesion (Saelman *et al.*, 1993), with α1(I) CB3 showing the greatest activity. Figure 3.1 illustrates the number of residues of the major peptides from human collagen α1(I) and collagen III after cleavage by cyanogen bromide.

The aim of this study was to isolate and purify the major CNBr peptides of Types I and III collagen and to test these fragments for their effects on cell behaviour. In this study cell proliferation and chemotaxis were examined.

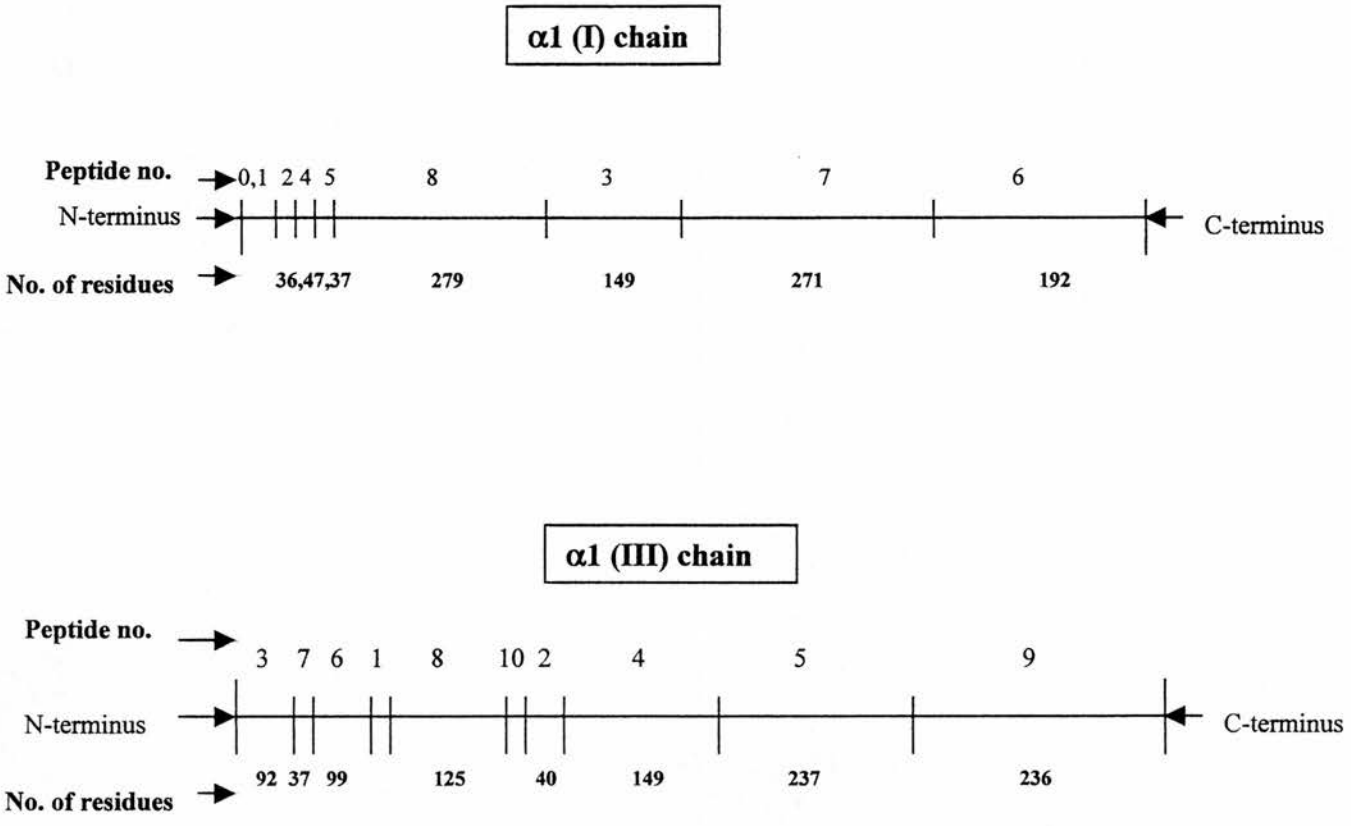

FIGURE 3.1: Diagram to illustrate the positions of the CNBr peptides in collagen α1(I) and α1(III) chains (Zijenah & Barnes 1990).
On top of the line the CNBr peptide number is shown, and below the line are the corresponding number of residues.

3.2 PURIFICATION AND CHARACTERISATION OF COLLAGENS I AND III

Collagens I and III are the predominant collagens in skin. Skin, therefore, can be used as an extremely inexpensive source of collagen, with high availability. Collagens I and III were isolated from porcine skin, as this skin was readily available and inexpensive. It is more difficult to obtain human skin in high amounts and also due to current health and safety considerations it was thought best to minimise the usage of human products as far as possible.

Collagens I and III were isolated from porcine skin (see Section 2.1) by a method of limited pepsin digestion and salt precipitation (Miller & Gay, 1982). The freeze-dried collagens were then analysed by SDS-PAGE on an 8% gel (Figure 3.2) and found to consist of the α1(I), α2(I) and α1(III) bands. In Figure 3.2 the α components from Type I and III collagens are shown. The β and ϒ components are also shown higher up the gel. In lane 3 of Figure 3.2, two bands were observed which correspond to α1(I) and α2(I) chains. In lane 4, one band was observed which corresponds to the α(III) chain. This is the same protein profile as seen by Bateman *et al.*, 1986.

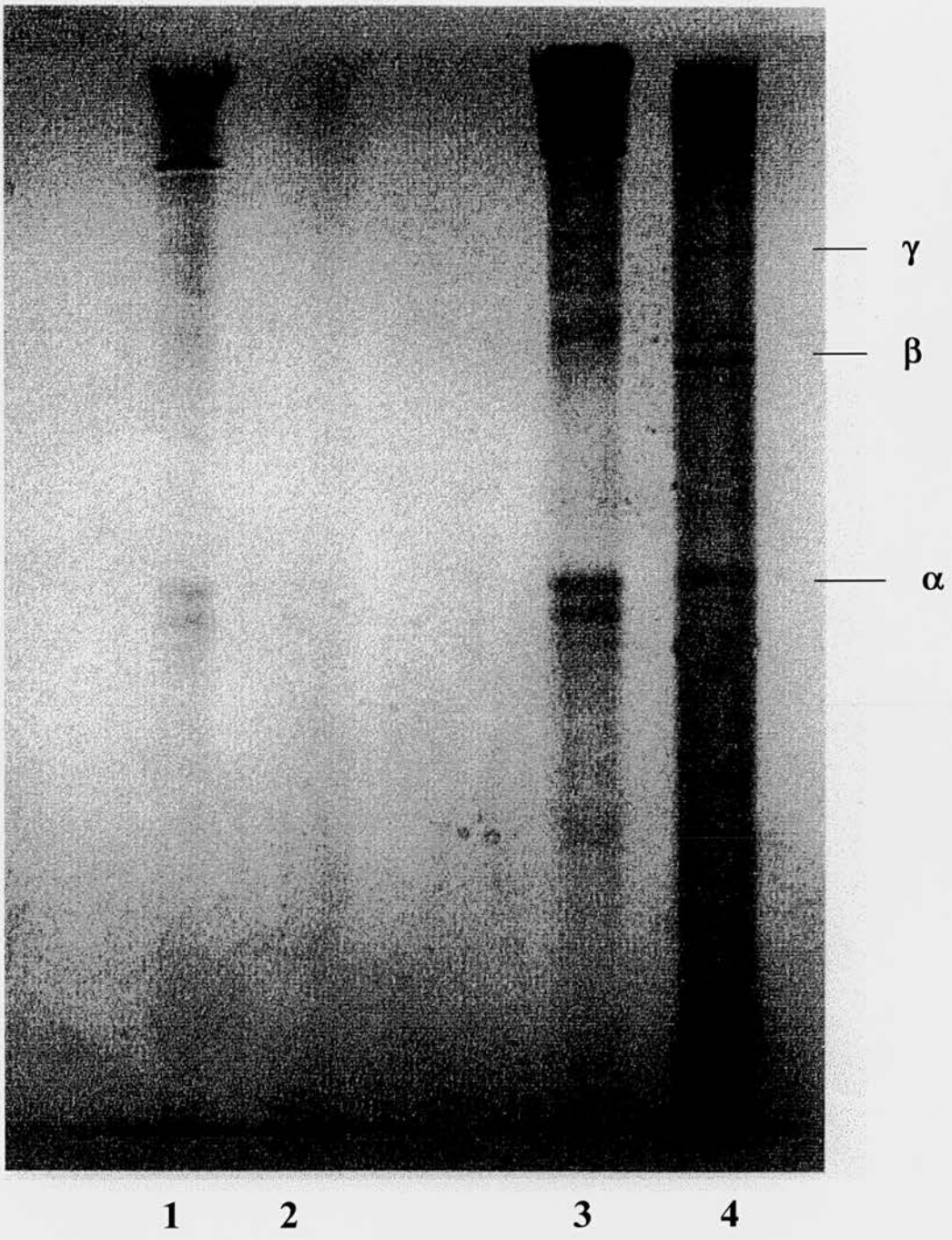

FIGURE 3.2: Separation of Collagens I and III by SDS-PAGE
Collagens I and III were isolated from porcine skin by pepsin digestion and salt precipitation (Miller & Gay, 1982). The separated collagens were analysed on SDS-PAGE. The α, β and γ components are shown.

Lanes Key:
1 – Type I (10μg)
2 – Type III (10μg)
3 – Type I (100μg)
4 – Type III (100μg)

3.3 DIGESTION BY CNBr

After the purification of Collagens I and III, proteins were digested with CNBr in order to provide peptides which could then be tested for their specific activities in this study.

The method in brief is as follows, Collagens I and III from pig skin (section 2.3.1), were dissolved in 70% formic acid (2mg collagen/ml acid) and then digested overnight in CNBr (1mg protein/2mg CNBr). Analysis by SDS-PAGE was carried out and results are shown in Figures 3.3 & 3.4

3.4 ISOLATION OF PEPTIDES

One of the aims of this study was to isolate and test the major collagen α1(I) CNBr peptides for their effects on cell proliferation and chemotaxis. To isolate the CNBr collagen-peptides, the CNBr digests were fractionated, by cation-exchange FPLC (Section 2.3.2) according to the method of Bateman *et al.* (1986). Results are shown in Figures 3.5 and 3.6. The first peak was due to unbound material passing through the column, whilst the last peak was due to undigested collagen being eluted. There was no need to separate the α1(I) and α2(I) chains before CNBr digestion since their CNBr-peptides have different molecular weights and would be separated after the FPLC (Bateman *et al.*, 1986). Quantitation of purified CNBr collagen peptides was based on the Sircol Collagen Assay (Section 2.3.3). Approx. 30-50μg of each CNBr peptide was purified per FPLC run.

Collagens I and III α chains have been successfully separated by CM-cellulose cation exchange chromatography by Piez *et al.*, (1963). However, this technique is tedious and the eluted chains and the peptides are excessively diluted so that fractions must be desalted and concentrated before further analysis. In contrast, a Mono-S cation-

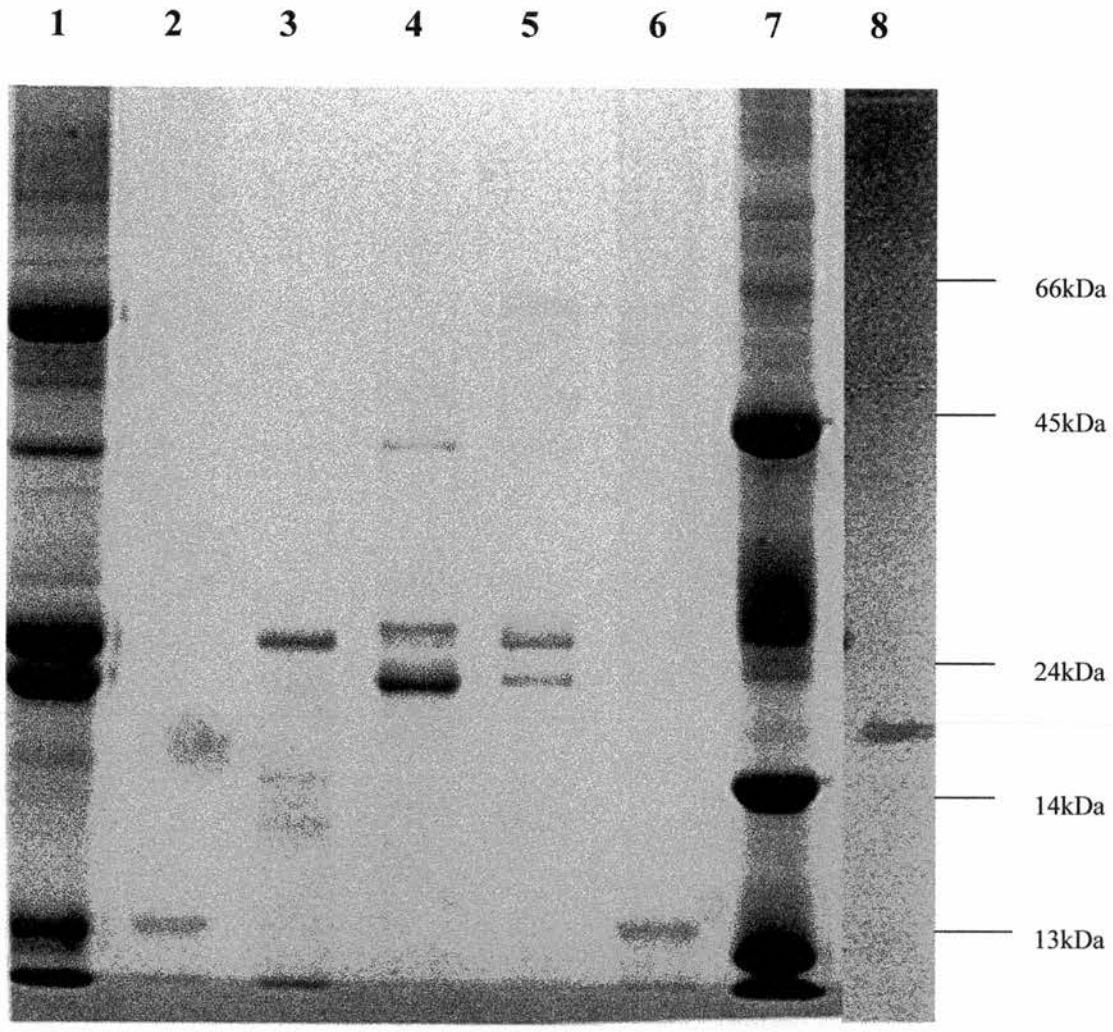

Figure 3.3: Separation of CNBr Peptides of Collagen I by SDS-PAGE
Collagen I was digested with CNBr and then fractionated by cation-exchange FPLC, as for method Bateman *et al.*, 1986. The separated CNBr peptides were then analysed on SDS-PAGE

Lane Key:
1 - Crude Digest
2+6 - CB3 -13kDa
3 - CB7 -25kDa
4 – CB8 -24kDa
5 – Crude Digest
7 – Molecular Weight Standards
8 – CB6 –17.5kDa

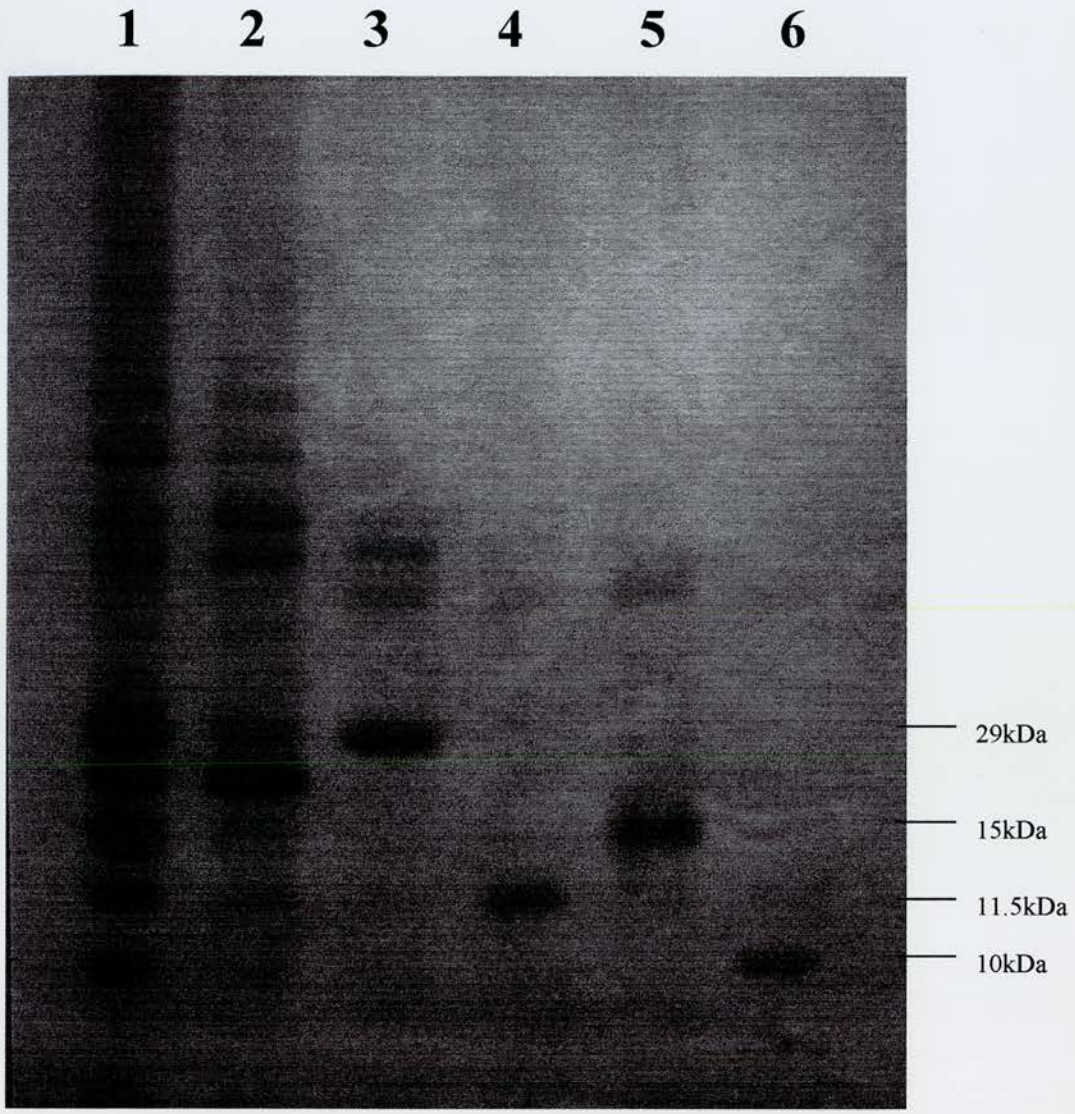

Figure 3.4: Separation of CNBr Peptides from Collagen III by SDS-PAGE
Collagen III was digested with CNBr and then fractionated by cation-exchange FPLC, as for method Bateman *et al.*, 1986. The separated CNBr peptides were analysed on SDS-PAGE.

Lanes Key:
1 – Crude Digest
2 – Crude Digest
3 – CB5 – 29kDa
4 – CB8 – 11.5kDa
5 - CB4 – 15kDa
6 – CB3 – 10kDa

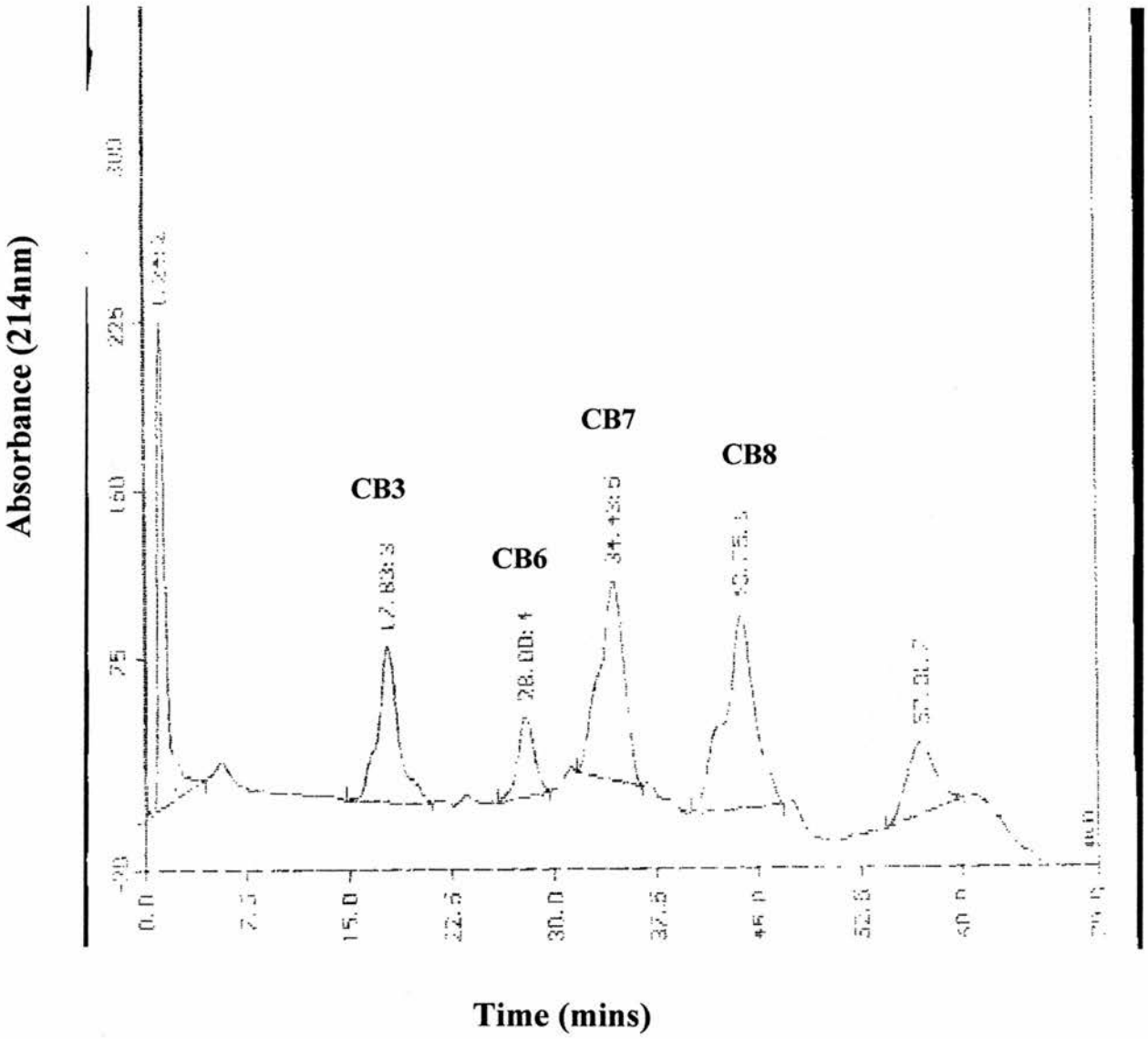

Figure 3.5: FPLC fractionation of porcine collagen α1(I) CNBr peptides by Mono S cation-exchange chromatography

0.5mg/ml of CNBr peptide was chromatographed at room temperature. A 65ml linear gradient of 0.07 to 0.026M NaCl in 0.02M sodium formate, pH 3.8, was delivered at a flow rate of 1ml/min. Absorbances were measured at 214nm.

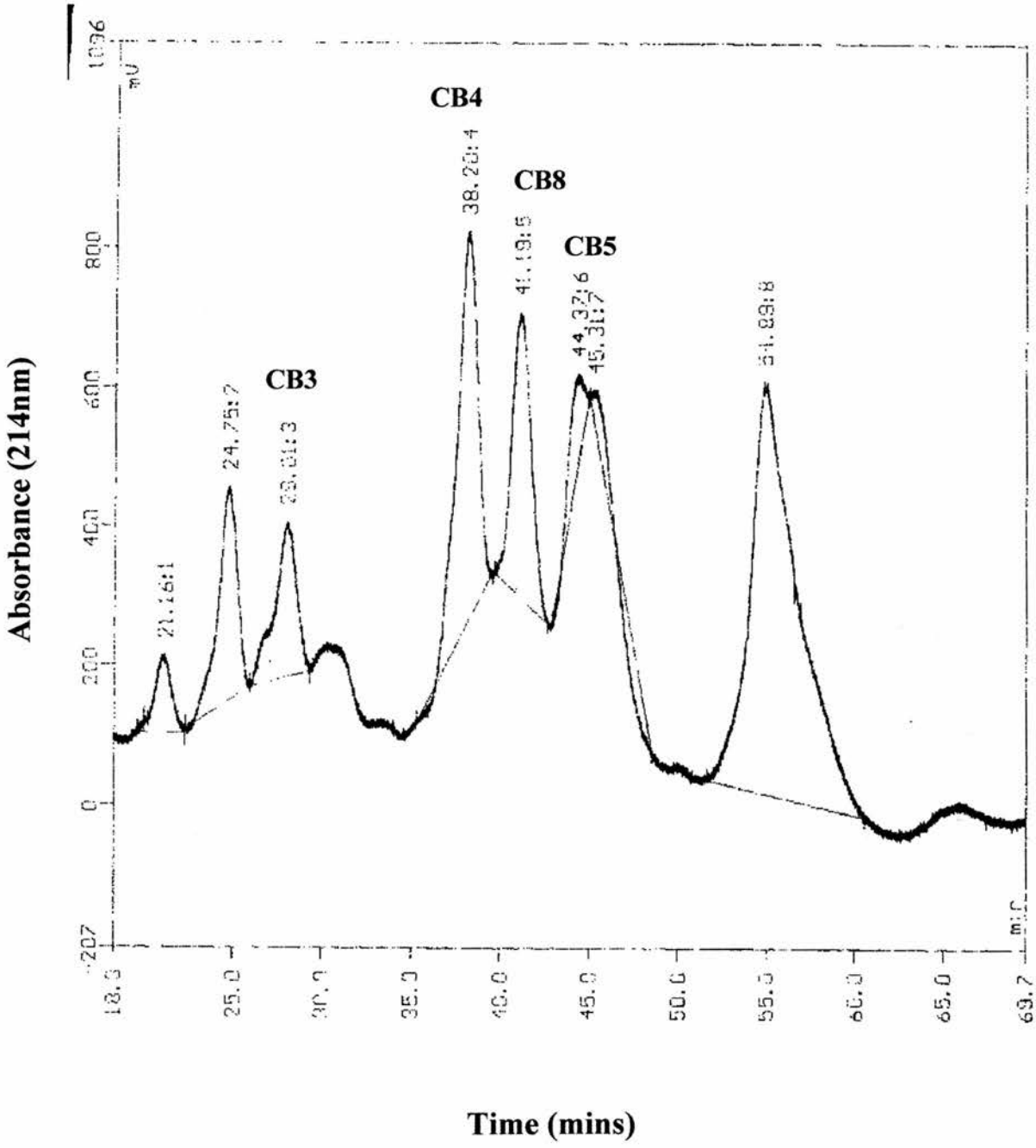

Figure 3.6: FPLC fractionation of porcine collagen α1(III) CNBr peptides by Mono S cation-exchange chromatography

0.5mg/ml of CNBr peptide was chromatographed at room temperature. A 65ml linear gradient of 0.07 to 0.026M NaCl in 0.02M sodium formate, pH 3.8, was delivered at a flow rate of 1ml/min. Absorbances were measured at 214nm.

exchange column resolves all the major peptides in a rapid and reproducible fashion (Bateman *et al.*, 1986). The difficulties of separation of CB7 and CB8 have been well documented (van der Rest & Fietzek 1982; van der Rest *et al.*, 1980, Miller *et al.*, 1983). Pure peptides were produced except for α1(I) CB7 and CB8, which also contained some incompletely cleaved fragments. These large partially cleaved peptides can be separated from CB7 and CB8 by gel permeation chromatography. The method used in these experiments has been shown by Bateman *et al.*, 1986, to give the best separation.

3.5 IDENTIFICATION OF PEPTIDES BY SDS-PAGE

Peptides were analysed by SDS-PAGE (12% acrylamide) to confirm the identity and purity of the separated CNBr peptides, as shown in Figures 3.3 & 3.4. Gels were dried and analysed by scanning densitometry, purity was approximately 80%.

3.6 EFFECTS ON CELL PROLIFERATION

The isolated CNBr fragments CB3, 6, 7, 8 from Type I collagen and CB3, 4, 5, 8 from Type III collagen (see Figures 3.3-3.6) were examined for their ability to stimulate both fibroblast (L929 cells and rat wound fibroblasts-RWF) and bovine aorta endothelial cell (BAEC) proliferation. The cell line L929 comes from an established cell line in mice and RWF cells come from a primary cell line. L929 is a modified and established cell line that is far-removed in character from its original cell origin. However, L929 cells are more robust and easy to handle and therefore were used in the setting-up and standardisation of the assays. Rat wound fibroblasts are nearer to the natural environment of wound healing, and were investigated in parallel.

Peptides at a range of concentrations (1pg-100μg/ml) were added to cell monolayers, which were then incubated over one day, two day and five day periods. It was decided to choose this range of concentrations with reference to previously published work (Postlethwaite *et al.*, 1978). Cells were set up in 96-well microplates for ease of handling and economy of scale. The numbers of cells present were quantified using the Methylene Blue dye-binding assay (Section 2.5.7; Oliver *et al.*, 1989).

The Methylene Blue dye-binding assay (Oliver *et al.*, 1989) was used to quantify cell numbers after growth in the presence of collagen-derived peptides. Although, this assay is reproducible, rapid, inexpensive and easy to perform, it does have limitations. The automated microplate photometer for reading optical densities of cells restricts the range of cell numbers that can be quantitated. As with all experiments, preliminary experiments to optimise the assay conditions (e.g. cell density and culture conditions) used were carried out.

The cells used in these experiments were grown in Dulbecco's Modified Eagle's Media (DMEM). Optimum growth conditions were obtained when the media was supplemented with 10% fetal calf serum (FCS). Therefore, the positive control was DMEM + 10% serum. Less serum (2%) was used in order to obtain slower growth. Cells grown in DMEM only i.e. Serum-free media (0% serum added) were used as negative controls. Cell growth is dependent upon seeding density and the concentration of serum in culture medium (Laurent *et al* 1989). In order to optimise cell culture conditions for this assay it was found that cells seeded at 5000 cells/well and cells supplemented with 2% serum gave the best conditions for assaying the effects of collagen peptides on cell proliferation. Cell growth in the presence of collagen peptides was calculated as a percentage of cell growth in DMEM containing 2% FCS. Cells grown in 10% serum give higher values, however to see an effect with these peptides, too high a count was obtained to measure in this study. Cells were overgrowing and detaching from the cell sheet. As optimal results were seen with 2% serum, this concentration was used as a standard. All results (including positive and

negative controls) are shown in Chapter 3.

3.6.1 Collagen I CNBr peptides [α1(I)]

Collagen I CNBr-derived peptides (in DMEM + 2% serum) were added to cells (L929, RWF and BAEC) and the effect on cell proliferation/growth studied over a 5 day period. The results from these experiments are shown below.

Cell proliferation in the presence of α1(I) CB8, on the three cell types was studied. Results for these experiments are recorded in Tables 3.1-3.3 and graphically shown in Figure 3.7. At 10μg/ml after 5 days, growth of both L929 and RWF cells was increased by almost 40%, while BAEC cell growth was increased by approximately 60%. These were highly statistically significant (Altman 1994). After 2 days, growth was increased by 53% in BAEC, 20% in RWF and 12% in L929 cells. However, after the first day of growth in the presence of CB8, growth in all three cell lines was inhibited. Growth in the presence of CB8 was concentration dependent, 10μg/ml giving optimum BAEC cell proliferation (Figure 3.8).

Cell proliferation in the presence of α1(I) CB3, on the three cell types was studied. Peptide CB3 at a concentration of 100μg/ml increased growth of both BAEC and RWF cells as shown in Tables 3.4-3.6 and graphically in Figure 3.9. Growth was increased by almost 20% after 5 days which was statistically significant. After 2 days, there was no increase in growth in any of the cell lines and after 1 day, growth was inhibited in all three cell lines. Growth in the presence of CB3 was concentration dependent, optimum growth occurring at 100μg/ml (Figure 3.10).

Peptide CB7 at a concentration of 100μg/ml increased cell growth in BEAC and RWF cells as shown in Tables 3.7-3.9 and these results are represented in graphical form in Figure 3.11. After 5 days, BAEC cell growth was increased by 14% and RWF growth was increased by 20%. Cell growth was not increased after 2 days in any of

BAEC	0% serum	2% serum	10% serum	2% serum +CB8
Day 1	0.745 (0.03)	0.903 (0.02)	1.450 (0.01)	0.799 (0.04)
Day 2	0.778 (0.02)	1.147 (0.02)	1.964 (0.01)	1.757 (0.05)
Day 5	1.010 (0.03)	1.384 (0.08)	2.114 (0.09)	2.220 (0.02)

Table 3.1: Effects of α1(I) CB8 on BAEC proliferation.
CB8 at 10μg/ml was added to cell line BAEC (in DMEM supplemented with 2 % serum) and growth after 1, 2 and 5 days was measured using the Methylene Blue assay. The data above represents absorbances at 630nm. Standard errors are shown (brackets). Control values are shown. Negative control - cells grown in DMEM only (0% serum). Positive control – cells grown in DMEM with 10% serum.

L929	0% serum	2% serum	10% serum	2% serum +CB8
Day 1	0.780 (0.04)	0.910 (0.03)	1.415 (0.09)	0.862 (0.03)
Day 2	0.825 (0.06)	1.050 (0.02)	1.995 (0.06)	1.171 (0.02)
Day 5	1.036 (0.01)	1.703 (0.04)	2.698 (0.06)	2.375 (0.06)

Table 3.2: Effects of α1(I) CB8 on L929 proliferation.
CB8 at 10μg/ml was added to L929 cells (in DMEM supplemented with 2 % serum) and growth after 1, 2 and 5 days was measured using the Methylene Blue assay. The data above represents absorbances at 630nm. Standard errors are shown (brackets). Control values are shown. Negative control - cells grown in DMEM only (0% serum). Positive control – cells grown in DMEM with 10% serum.

RWF	0% serum	2% serum	10% serum	2% serum +CB8
Day 1	0.301 (0.02)	0.547 (0.04)	0.899 (0.06)	0.420 (0.09)
Day 2	0.336 (0.03)	0.610 (0.04)	0.980 (0.03)	0.730 (0.03)
Day 5	0.480 (0.06)	0.950 (0.04)	1.504 (0.07)	1.30 (0.04)

Table 3.3: Effects of α1(I) CB8 on RWF proliferation.
CB8 at 10μg/ml was added to RWF cells (in DMEM supplemented with 2 % serum) and growth after 1, 2 and 5 days was measured using the Methylene Blue assay. The data above represents absorbances at 630nm. Standard errors are shown (brackets). Control values are shown. Negative control - cells grown in DMEM only (0% serum). Positive control – cells grown in DMEM with 10% serum.

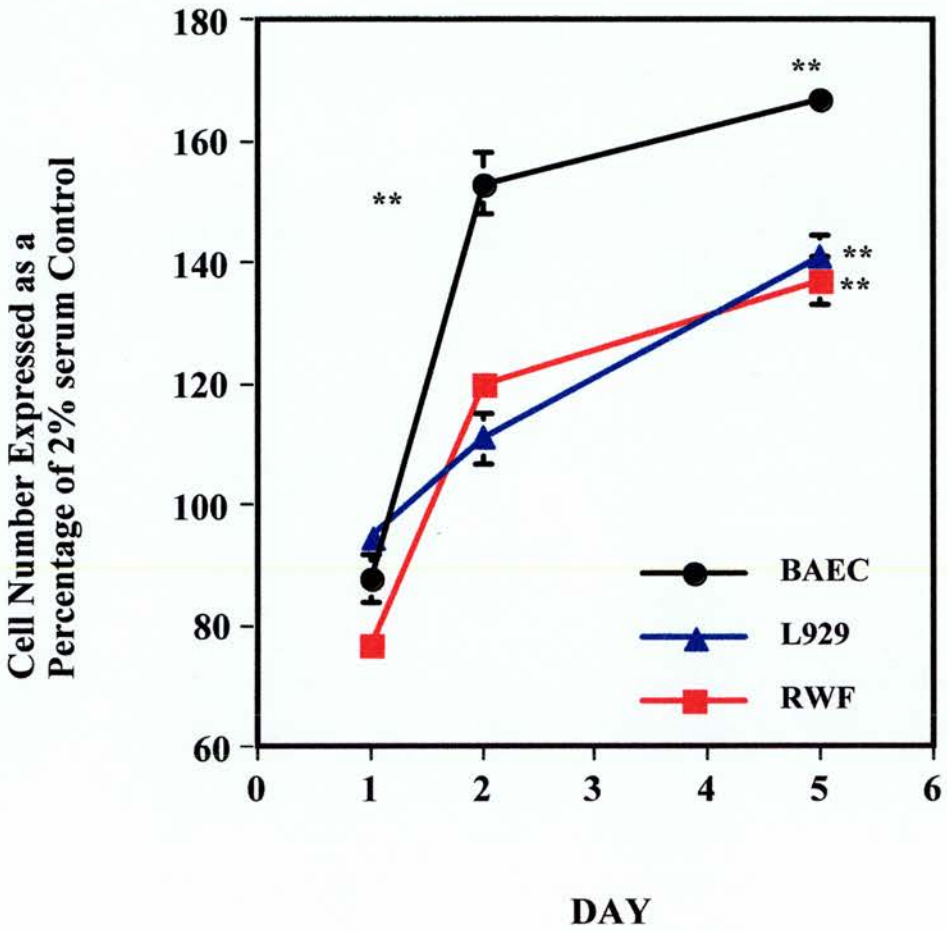

FIGURE 3.7: Effects of α1(I) CB8 on cell proliferation

The cell lines used were BAEC, L929 and RWF. Peptide CB8 was added at a concentration of 10μg/ml and the growth of the three cell types was measured over 5 days. Cells were seeded at a density of 5000 cells/well in 96-microwell plates containing 100μl of DMEM + 2% FCS. Each value represents the mean of 6 wells (Student's t-Test). Standard errors are shown, *$p<0.05$, **$p<0.01$.

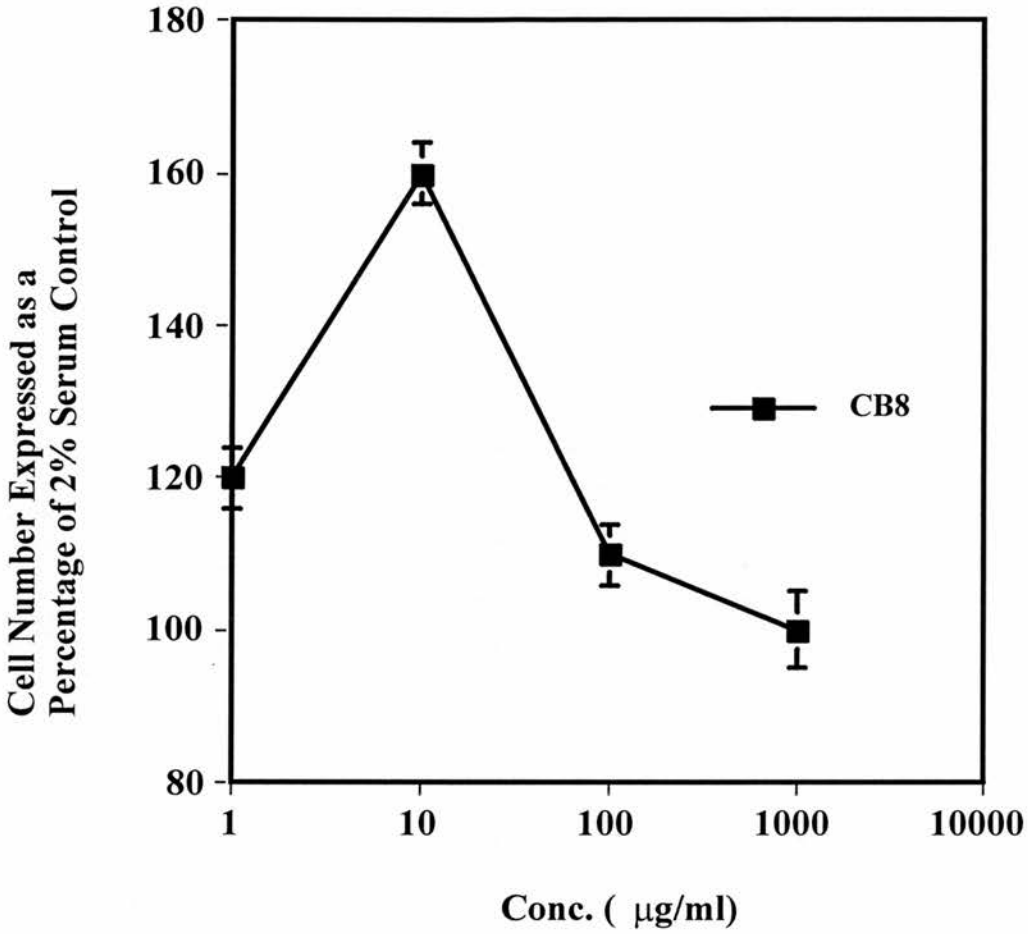

FIGURE 3.8: Dependence of BAEC proliferation on the concentration of α1(I) CB8

Optimum proliferation (after 5 days) of BAEC occurs at a peptide concentration of 10μg/ml. Cells were seeded at a density of 5000 cells/well in 96-microwell plates containing 100μl of DMEM + 2% FCS. Each value represents the mean of 6 wells (Student's t-Test). Standard errors are shown.

BAEC	0% serum	2% serum	10% serum	2% serum +CB3
Day 1	0.690 (0.01)	0.803 (0.01)	1.185 (0.02)	0.676 (0.02)
Day 2	0.700 (0.02)	0.829 (0.02)	1.272 (0.02)	0.800 (0.02)
Day 5	0.998 (0.03)	1.46 (0.02)	2.008 (0.02)	1.697 (0.02)

Table 3.4: Effects of α1(I) CB3 on BAEC proliferation.
CB3 at 100μg/ml was added to cell line BAEC (in DMEM supplemented with 2 % serum) and growth after 1, 2 and 5 days was measured using the Methylene Blue assay. The data above represents absorbances at 630nm. Standard errors are shown (brackets). Control values are shown. Negative control - cells grown in DMEM only (0% serum). Positive control – cells grown in DMEM with 10% serum.

L929	0% serum	2% serum	10% serum	2% serum +CB3
Day 1	0.894 (0.01)	1.200 (0.02)	1.576 (0.03)	1.117 (0.02)
Day 2	0.921 (0.02)	1.379 (0.02)	2.115 (0.05)	1.35 (0.03)
Day 5	1.201 (0.03)	2.033 (0.06)	2.424 (0.1)	2.012 (0.09)

Table 3.5: Effects of α1(I) CB3 on L929 proliferation.
CB3 at 100μg/ml was added to L929 cells (in DMEM supplemented with 2 % serum) and growth after 1, 2 and 5 days was measured using the Methylene Blue assay. The data above represents absorbances at 630nm. Standard errors are shown (brackets). Control values are shown. Negative control - cells grown in DMEM only (0% serum). Positive control – cells grown in DMEM with 10% serum

RWF	0% serum	2% serum	10% serum	2% serum +CB3
Day 1	0.315 (0.02)	0.519 (0.01)	0.851 (0.1)	0.417 (0.02)
Day 2	0.325 (0.03)	0.623 (0.03)	0.940 (0.03)	0.622 (0.03)
Day 5	0.450 (0.02)	0.902 (0.02)	1.24 (0.05)	1.08 (0.03)

Table 3.6: Effects of α1(I) CB3 on RWF proliferation.
CB3 at 100μg/ml was added to RWF cells (in DMEM supplemented with 2 % serum) and growth after 1, 2 and 5 days was measured using the Methylene Blue assay. The data above represents absorbances at 630nm. Standard errors are shown (brackets). Control values are shown. Negative control - cells grown in DMEM only (0% serum). Positive control – cells grown in DMEM with 10% serum

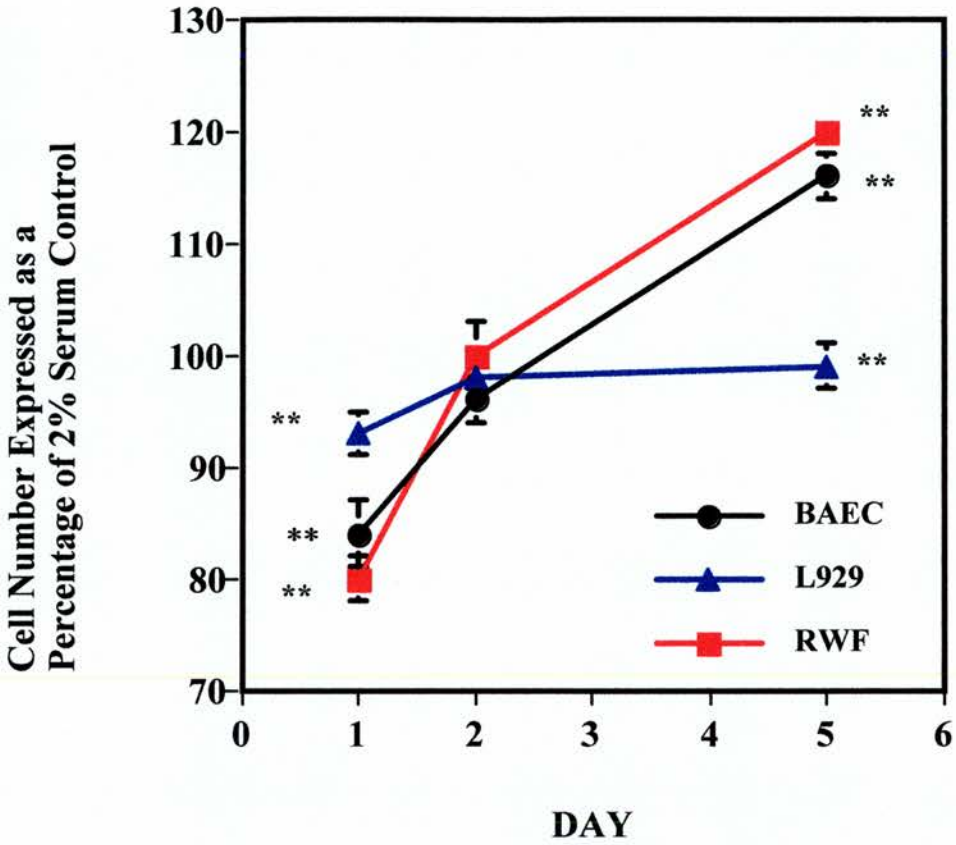

FIGURE 3.9: Effects of α1(I) CB3 on cell proliferation

The cell lines used were BAEC, L929 and RWF. Peptide CB3 was added at a concentration of 100μg/ml and the growth of the three cell types was measured over 5 days. Cells were seeded at a density of 5000 cells/well in 96-microwell plates containing 100μl of DMEM + 2% FCS. Each value represents the mean of 6 wells (Student's t-Test). Standard errors are shown, *$p<0.05$, **$p<0.01$.

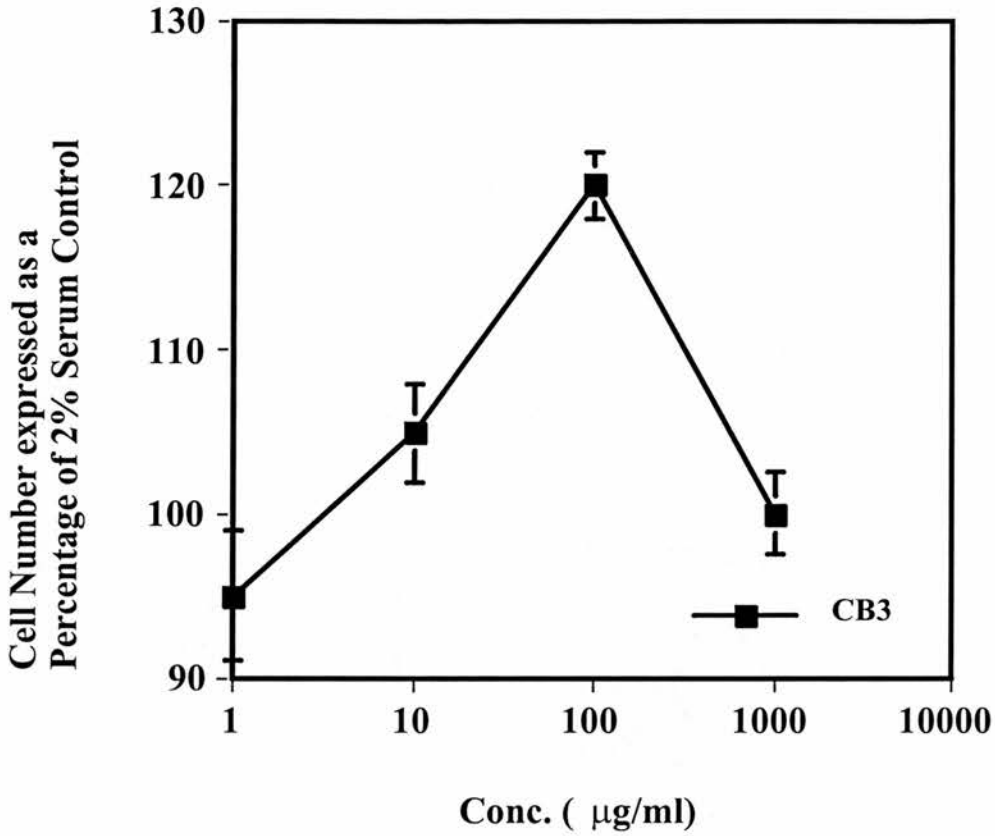

FIGURE 3.10: Dependence of BAEC proliferation on the concentration of α1(I) CB3

Optimum proliferation (after 5 days) of BAEC occurs at a peptide concentration of 100μg/ml. Cells were seeded at a density of 5000 cells/well in 96-microwell plates containing 100μl of DMEM + 2% FCS. Each value represents the mean of 6 wells (Student's t-Test). Standard errors are shown.

BAEC	0% serum	2% serum	10% serum	2% serum +CB7
Day 1	0.319 (0.04)	0.430 (0.07)	1.175 (0.07)	0.324 (0.01)
Day 2	0.400 (0.02)	0.597 (0.03)	1.211 (0.05)	0.464 (0.03)
Day 5	0.531 (0.02)	0.921 (0.06)	1.527 (0.07)	1.054 (0.05)

Table 3.7: Effects of α1(I) CB7 on BAEC proliferation.
CB7 at 100μg/ml was added to cell line BAEC (in DMEM supplemented with 2 % serum) and growth after 1, 2 and 5 days was measured using the Methylene Blue assay. The data above represents absorbances at 630nm. Standard errors are shown (brackets). Control values are shown. Negative control - cells grown in DMEM only (0% serum). Positive control – cells grown in DMEM with 10% serum.

L929	0% serum	2% serum	10% serum	2% serum +CB7
Day 1	0.347 (0.01)	0.610 (0.02)	1.262 (0.03)	0.611 (0.04)
Day 2	0.468 (0.03)	0.630 (0.01)	1.277 (0.03)	0.669 (0.01)
Day 5	0.724 (0.06)	1.441 (0.07)	2.485 (0.04)	1.457 (0.06)

Table 3.8: Effects of α1(I) CB7 on L929 proliferation.
CB7 at 100μg/ml was added to L929 cells (in DMEM supplemented with 2 % serum) and growth after 1, 2 and 5 days was measured using the Methylene Blue assay. The data above represents absorbances at 630nm. Standard errors are shown (brackets). Control values are shown. Negative control - cells grown in DMEM only (0% serum). Positive control – cells grown in DMEM with 10% serum.

RWF	0% serum	2% serum	10% serum	2% serum +CB7
Day 1	0.349 (0.01)	0.578 (0.05)	1.204 (0.07)	0.560 (0.01)
Day 2	0.467 (0.03)	0.869 (0.02)	1.999 (0.05)	0.978 (0.02)
Day 5	0.692 (0.04)	1.807 (0.06)	2.455 (0.04)	2.158 (0.02)

Table 3.9: Effects of α1(I) CB7 on RWF proliferation.
CB7 at 100μg/ml was added to RWF cells (in DMEM supplemented with 2 % serum) and growth after 1, 2 and 5 days was measured using the Methylene Blue assay. The data above represents absorbances at 630nm. Standard errors are shown (brackets). Control values are shown. Negative control - cells grown in DMEM only (0% serum). Positive control – cells grown in DMEM with 10% serum.

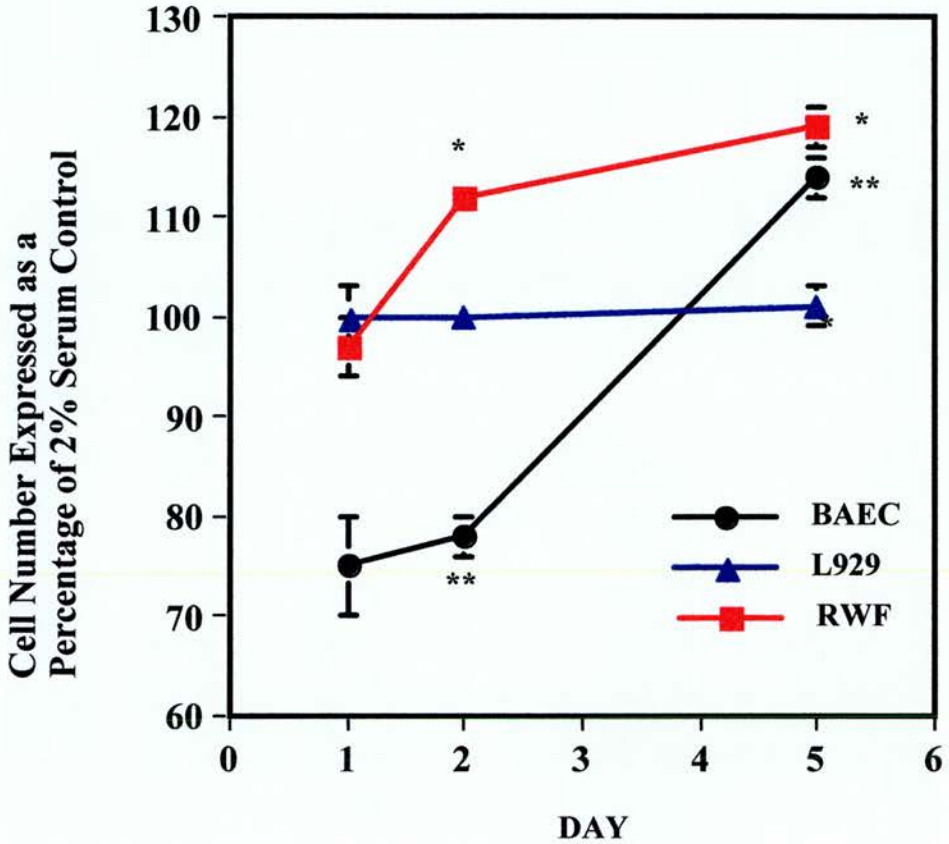

FIGURE 3.11: Effects of α1(I) CB7 on cell proliferation
The cell lines used were BAEC, L929 and RWF. Peptide CB7 was added at a concentration of 100μg/ml and the growth of the three cell types was measured over 5 days. Cells were seeded at a density of 5000 cells/well in 96-microwell plates containing 100μl of DMEM + 2% FCS. Each value represents the mean of 6 wells (Student's t-Test). Standard errors are shown, *$p<0.05$, **$p<0.01$.

the cell lines. After day one, BAEC cell growth was inhibited.

Peptide CB6 had no significant effect on cell proliferation/growth on any of the cell types and at any of the concentrations tested. Results are shown in Tables 3.10-3.12. Figure 3.12 shows the effect of CB6 at 100μg/ml.

3.6.2 Collagen III CNBr peptides [α1(III)]

Similarly, Collagen III CNBr-derived peptides were examined for their effects on the proliferation/growth of BAEC, L929 and RWF cell lines. The results are shown below.

Peptide CB4 significantly stimulated cell growth in L929 and BAEC cells as shown in Tables 3.13-3.15 and Figure 3.13. At a concentration of 1ng/ml after 5 days of growth, L929 growth was stimulated by 35% and BAEC cells stimulated by 13% (Figure 3.13). This was highly significant. The effect was concentration dependent (Figure 3.14), proliferation being optimum at 1ng/ml. Higher concentrations were inhibitory. RWF cell growth was not increased after 5 days. After 2 days, RWF and BAEC growth was inhibited. After day 1, all three cell line's growth was inhibited (Figure 3.13).

CB8 increased growth of all cell types tested as shown in Tables 3.16-3.18 and Figure 3.15. After 5 days, BAEC growth increased by 19%, RWF growth increased by 15% and L929 growth increased by 13%. Growth was inhibited in all three cell lines after days 1 and 2. Growth of BAEC cells was concentration-dependent, optimum growth occurring at a concentration of 1ng/ml (Figure 3.16).

CB5 and CB3 had no biologically significant effects on cell proliferation/growth as shown in Tables 3.19-3.24 and Figures 3.17 & 3.18.

BAEC	0% serum	2% serum	10% serum	2% serum +CB6
Day 1	0.451 (0.01)	0.648 (0.03)	1.200 (0.02)	0.618 (0.04)
Day 2	0.563 (0.03)	0.860 (0.02)	1.272 (0.02)	0.811 (0.04)
Day 5	0.885 (0.06)	1.599 (0.02)	2.008 (0.02)	1.598 (0.01)

Table 3.10: Effects of α1(I) CB6 on BAEC proliferation.
CB6 at 100μg/ml was added to cell line BAEC (in DMEM supplemented with 2 % serum) and growth after 1, 2 and 5 days was measured using the Methylene Blue assay. The data above represents absorbances at 630nm. Standard errors are shown (brackets). Control values are shown. Negative control - cells grown in DMEM only (0% serum). Positive control – cells grown in DMEM with 10% serum.

L929	0% serum	2% serum	10% serum	2% serum +CB6
Day 1	0.359 (0.06)	0.617 (0.01)	1.216 (0.04)	0.650 (0.03)
Day 2	0.480 (0.03)	0.793 (0.02)	1.452 (0.02)	0.855 (0.09)
Day 5	0.703 (0.01)	1.514 (0.04)	2.289 (0.05)	1.595 (0.03)

Table 3.11: Effects of α1(I) CB6 on L929 proliferation.
CB6 at 100μg/ml was added to L929 cells (in DMEM supplemented with 2 % serum) and growth after 1, 2 and 5 days was measured using the Methylene Blue assay. The data above represents absorbances at 630nm. Standard errors are shown (brackets). Control values are shown. Negative control - cells grown in DMEM only (0% serum). Positive control – cells grown in DMEM with 10% serum.

RWF	0% serum	2% serum	10% serum	2% serum +CB6
Day 1	0.325 (0.02)	0.469 (0.01)	0.948 (0.05)	0.450 (0.05)
Day 2	0.415 (0.02)	0.597 (0.07)	1.175 (0.2)	0.597 (0.02)
Day 5	0.589 (0.04)	0.942 (0.04)	1.492 (0.06)	0.985 (0.09)

Table 3.12: Effects of α1(I) CB6 on RWF proliferation.
CB6 at 100μg/ml was added to RWF cells (in DMEM supplemented with 2 % serum) and growth after 1, 2 and 5 days was measured using the Methylene Blue assay. The data above represents absorbances at 630nm. Standard errors are shown (brackets). Control values are shown. Negative control - cells grown in DMEM only (0% serum). Positive control – cells grown in DMEM with 10% serum.

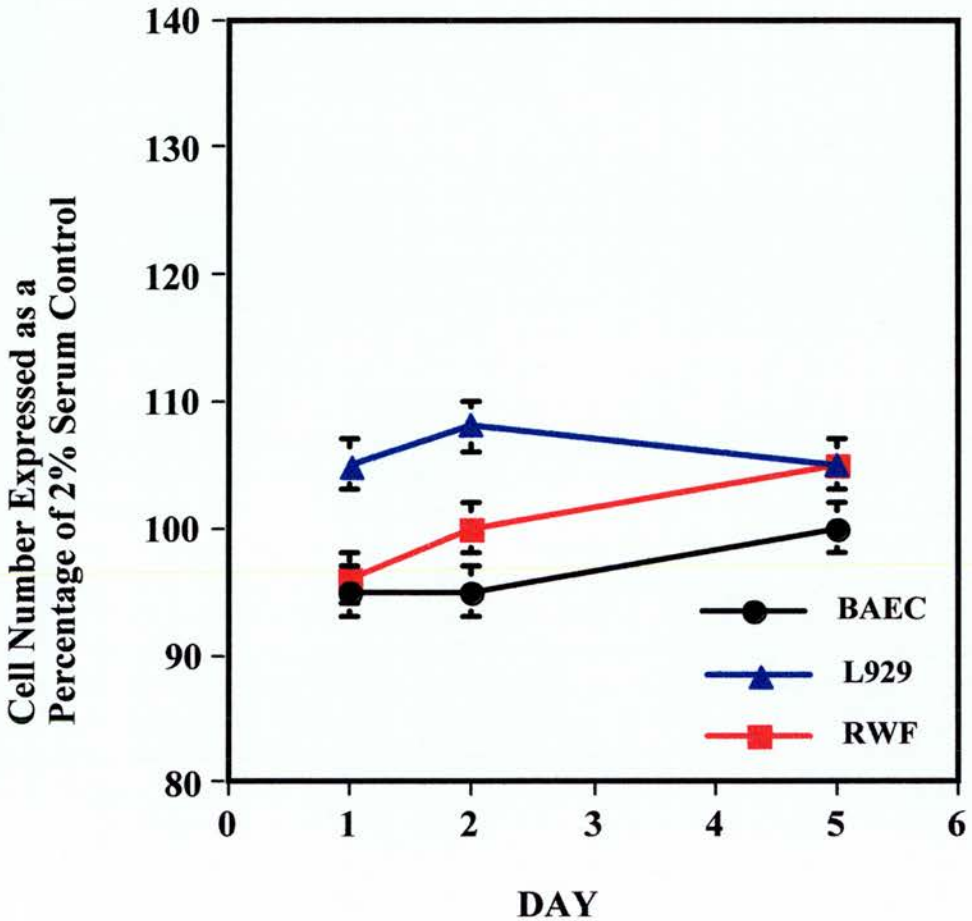

FIGURE 3.12: Effects of α1(I) CB6 on cell proliferation
The cell lines used were BAEC, L929 and RWF. Peptide CB6 was added at a concentration of 100μg/ml and the growth of the three cell types was measured over 5 days. Cells were seeded at a density of 5000 cells/well in 96-microwell plates containing 100μl of DMEM + 2% FCS. Each value represents the mean of 6 wells (Student's t-Test). Standard errors are shown, *$p<0.05$, **$p<0.01$.

BAEC	0% serum	2% serum	10% serum	2% serum +CB4
Day 1	0.423 (0.01)	0.729 (0.01)	1.135 (0.02)	0.524 (0.01)
Day 2	0.510 (0.02)	0.835 (0.02)	1.329 (0.03)	0.750 (0.02)
Day 5	0.563 (0.01)	1.505 (0.03)	2.015 (0.04)	1.700 (0.03)

Table 3.13: Effects of α1(III) CB4 on BAEC proliferation.
CB4 at 1ng/ml was added to cell line BAEC (in DMEM supplemented with 2 % serum) and growth after 1, 2 and 5 days was measured using the Methylene Blue assay. The data above represents absorbances at 630nm. Standard errors are shown (brackets). Control values are shown. Negative control - cells grown in DMEM only (0% serum). Positive control – cells grown in DMEM with 10% serum.

L929	0% serum	2% serum	10% serum	2% serum + CB4
Day 1	0.526 (0.01)	0.685 (0.01)	1.213 (0.03)	0.575 (0.01)
Day 2	0.627 (0.03)	0.821 (0.02)	1.456 (0.04)	1.020 (0.02)
Day 5	0.698 (0.02)	1.426 (0.03)	2.231 (0.04)	1.930 (0.04)

Table 3.14: Effects of α1(III) CB4 on L929 proliferation.
CB4 at 1ng/ml was added to L929 cells (in DMEM supplemented with 2 % serum) and growth after 1, 2 and 5 days was measured using the Methylene Blue assay. The data above represents absorbances at 630nm. Standard errors are shown (brackets). Control values are shown. Negative control - cells grown in DMEM only (0% serum). Positive control – cells grown in DMEM with 10% serum.

RWF	0% serum	2% serum	10% serum	2% serum +CB4
Day 1	0.410 (0.01)	0.596 (0.01)	1.195 (0.04)	0.538 (0.01)
Day 2	0.491 (0.01)	0.706 (0.01)	1.309 (0.05)	0.651 (0.01)
Day 5	0.526 (0.03)	1.342 (0.03)	2.016 (0.03)	1.341 (0.03)

Table 3.15: Effects of α1(III) CB4 on RWF proliferation.
CB4 at 1ng/ml was added to RWF cells (in DMEM supplemented with 2 % serum) and growth after 1, 2 and 5 days was measured using the Methylene Blue assay. The data above represents absorbances at 630nm. Standard errors are shown (brackets). Control values are shown. Negative control - cells grown in DMEM only (0% serum). Positive control – cells grown in DMEM with 10% serum.

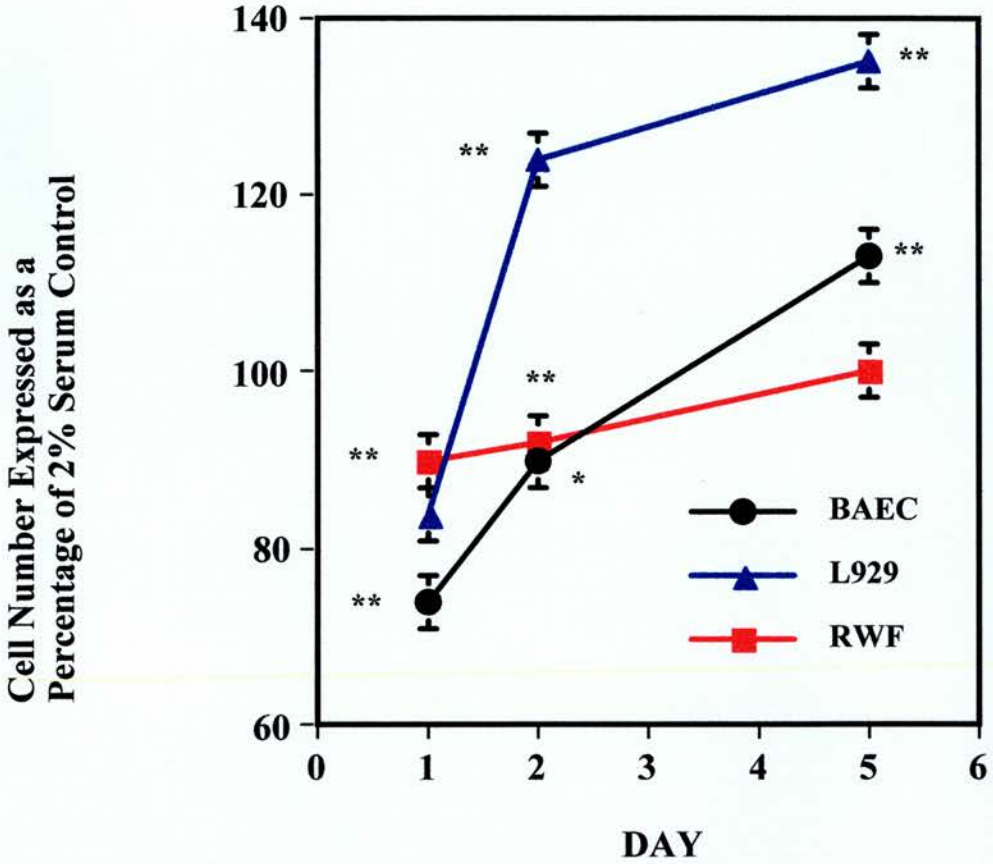

FIGURE 3.13: Effects of α1(III) CB4 on cell proliferation
The cell lines used were BAEC, L929 and RWF. Peptide CB4 was added at a concentration of 1ng/ml and the growth of the three cell types was measured over 5 days. Cells were seeded at a density of 5000 cells/well in 96-microwell plates containing 100µl of DMEM + 2% FCS. Each value represents the mean of 6 wells (Student's t-Test). Standard errors are shown, *$p<0.05$, **$p<0.01$.

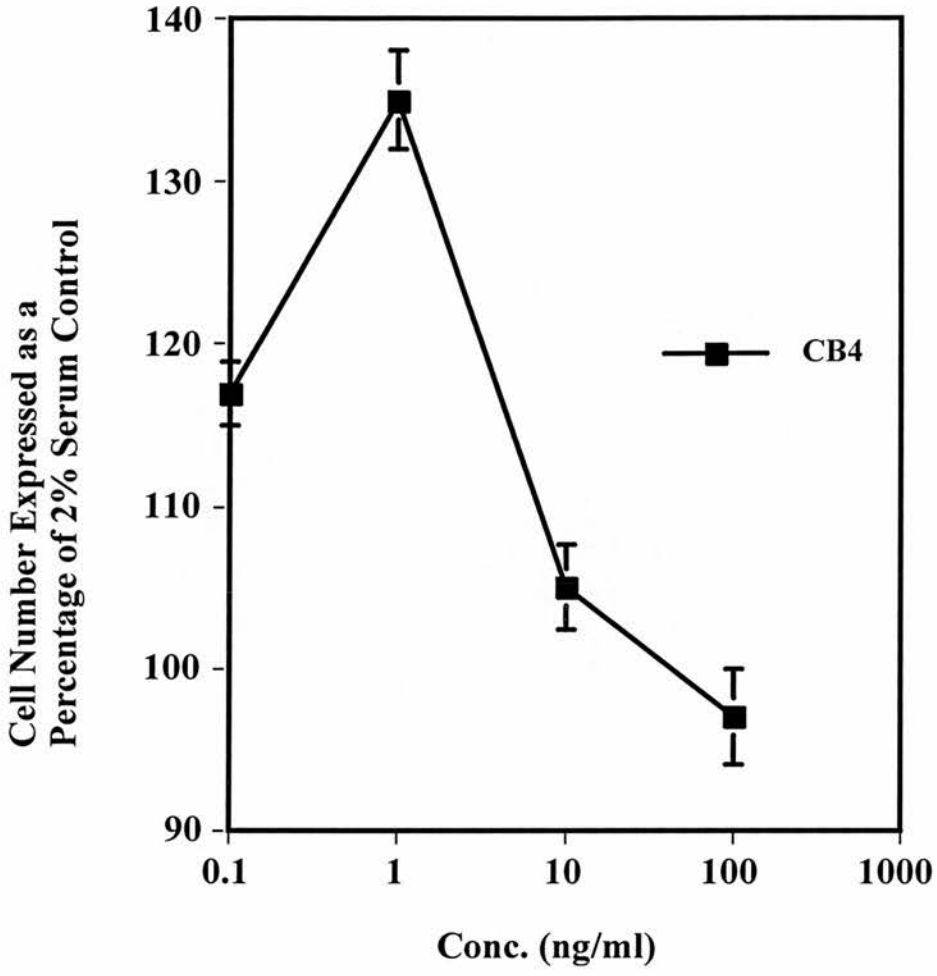

FIGURE 3.14: Dependence of L929 proliferation on the concentration of α1(III) CB4

Optimum proliferation (after 5 days) of L929 cells occurs at a peptide concentration of 1ng/ml. Cells were seeded at a density of 5000 cells/well in 96-microwell plates containing 100µl of DMEM + 2% FCS. Each value represents the mean of 6 wells (Student's t-Test). Standard errors are shown.

BAEC	0% serum	2% serum	10% serum	2% serum +CB8
Day 1	0.481 (0.01)	0.674 (0.01)	1.212 (0.02)	0.512 (0.01)
Day 2	0.534 (0.03)	0.801 (0.01)	1.493 (0.03)	0.783 (0.02)
Day 5	0.892 (0.03)	1.523 (0.03)	2.314 (0.02)	1.806 (0.03)

Table 3.16: Effects of α1(III) CB8 on BAEC proliferation.
CB8 at 1ng/ml was added to cell line BAEC (in DMEM supplemented with 2 % serum) and growth after 1, 2 and 5 days was measured using the Methylene Blue assay. The data above represents absorbances at 630nm. Standard errors are shown (brackets). Control values are shown. Negative control - cells grown in DMEM only (0% serum). Positive control – cells grown in DMEM with 10% serum.

L929	0% serum	2% serum	10% serum	2% serum +CB8
Day 1	0.499 (0.05)	0.624 (0.01)	1.201 (0.04)	0.555 (0.01)
Day 2	0.583 (0.03)	0.794 (0.02)	1.302 (0.02)	0.741 (0.02)
Day 5	0.821 (0.01)	1.499 (0.03)	2.018 (0.05)	1.701 (0.02)

Table 3.17: Effects of α1(III) CB8 on L929 proliferation.
CB8 at 1ng/ml was added to L929 cells (in DMEM supplemented with 2 % serum) and growth after 1, 2 and 5 days was measured using the Methylene Blue assay. The data above represents absorbances at 630nm. Standard errors are shown (brackets). Control values are shown. Negative control - cells grown in DMEM only (0% serum). Positive control – cells grown in DMEM with 10% serum

RWF	0% serum	2% serum	10% serum	2% serum +CB8
Day 1	0.333 (0.02)	0.521 (0.01)	0.986 (0.04)	0.443 (0.01)
Day 2	0.402 (0.02)	0.600 (0.01)	1.149 (0.03)	0.544 (0.01)
Day 5	0.516 (0.03)	0.956 (0.02)	1.483 (0.04)	1.101 (0.02)

Table 3.18: Effects of α1(III) CB8 on RWF proliferation.
CB8 at 1ng/ml was added to RWF cells (in DMEM supplemented with 2 % serum) and growth after 1, 2 and 5 days was measured using the Methylene Blue assay. The data above represents absorbances at 630nm. Standard errors are shown (brackets). Control values are shown. Negative control - cells grown in DMEM only (0% serum). Positive control – cells grown in DMEM with 10% serum

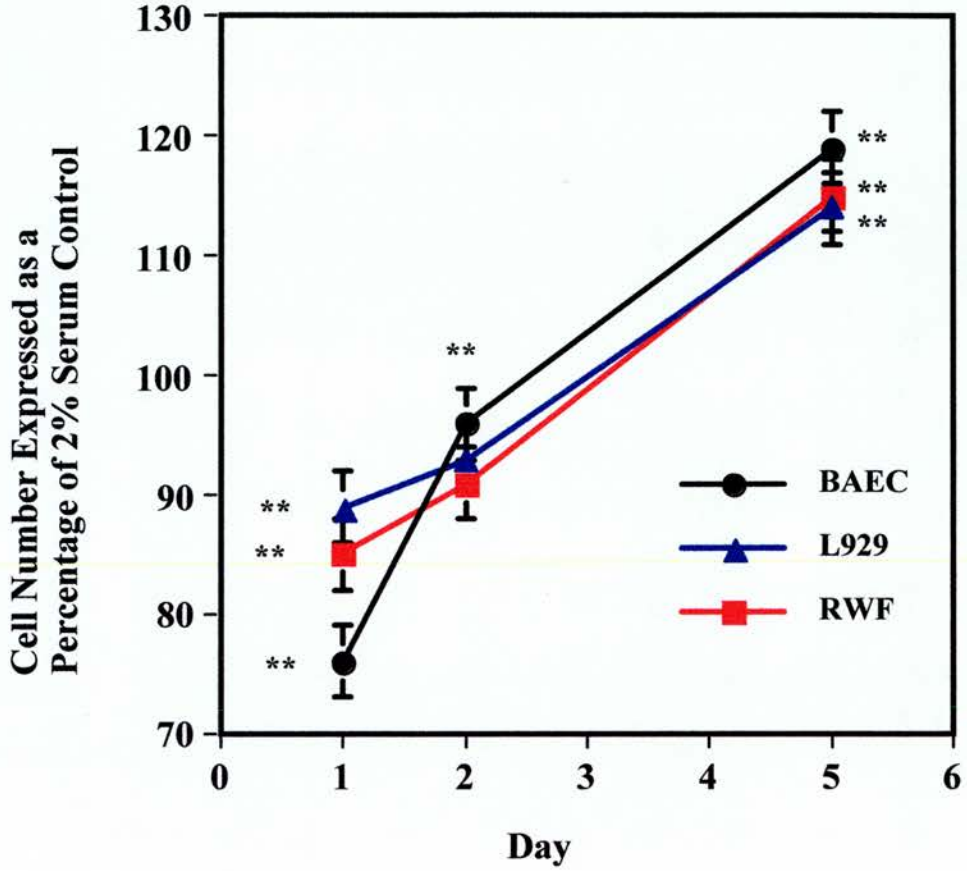

FIGURE 3.15: Effects of α1(III) CB8 on cell proliferation
The cell lines used were BAEC, L929 and RWF. Peptide CB8 was added at a concentration of 1ng/ml and the growth of the three cell types was measured over 5 days. Cells were seeded at a density of 5000 cells/well in 96-microwell plates containing 100µl of DMEM + 2% FCS. Each value represents the mean of 6 wells (Student's t-Test). Standard errors are shown, *$p<0.05$, **$p<0.01$.

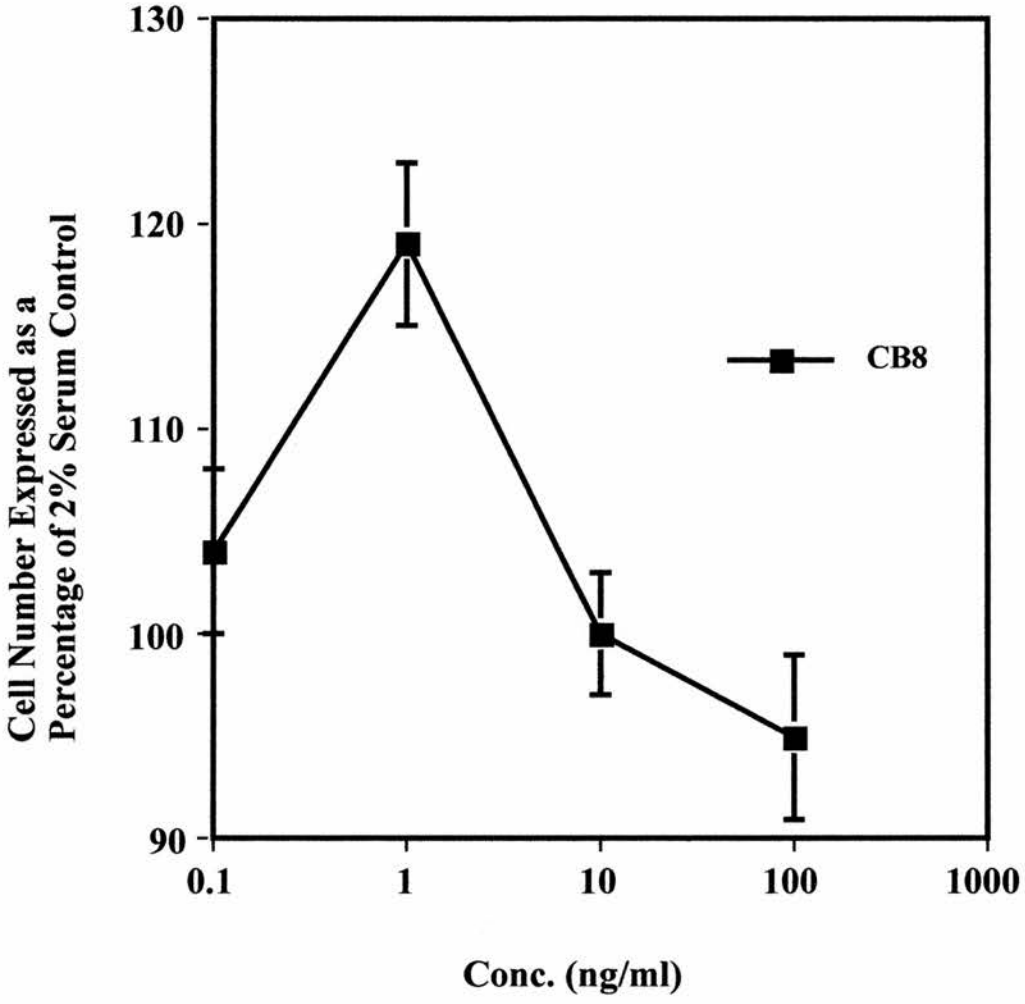

FIGURE 3.16: Dependence of BAEC proliferation on the concentration of α1(III) CB8

Optimum proliferation (after 5 days) of BAEC occurs at a peptide concentration of 1ng/ml. Cells were seeded at a density of 5000 cells/well. Cells were grown in microwell plates containing 100µl of DMEM + 2% FCS. Each value represent the mean of 6 wells (student's t-Test). Standard errors are shown.

BAEC	0% serum	2% serum	10% serum	2% serum +CB3
Day 1	0.435 (0.02)	0.681 (0.01)	1.198 (0.03)	0.677 (0.01)
Day 2	0.529 (0.02)	0.793 (0.02)	1.272 (0.03)	0.793 (0.02)
Day 5	0.821 (0.03)	1.509 (0.03)	2.001 (0.06)	1.572 (0.03)

Table 3.19: Effects of α1(III) CB3 on BAEC proliferation.
CB3 at 100μg/ml was added to cell line BAEC (in DMEM supplemented with 2 % serum) and growth after 1, 2 and 5 days was measured using the Methylene Blue assay. The data above represents absorbances at 630nm. Standard errors are shown (brackets). Control values are shown. Negative control - cells grown in DMEM only (0% serum). Positive control – cells grown in DMEM with 10% serum.

L929	0% serum	2% serum	10% serum	2% serum +CB3
Day 1	0.484 (0.02)	0.607 (0.01)	1.229 (0.04)	0.623 (0.01)
Day 2	0.539 (0.01)	0.783 (0.01)	1.418 (0.04)	0.813 (0.02)
Day 5	0.818 (0.03)	1.525 (0.03)	2.204 (0.06)	1.573 (0.03)

Table 3.20: Effects of α1(III) CB3 on L929 proliferation.
CB3 at 100μg/ml was added to L929 cells (in DMEM supplemented with 2 % serum) and growth after 1, 2 and 5 days was measured using the Methylene Blue assay. The data above represents absorbances at 630nm. Standard errors are shown (brackets). Control values are shown. Negative control - cells grown in DMEM only (0% serum). Positive control – cells grown in DMEM with 10% serum.

RWF	0% serum	2% serum	10% serum	2% serum +CB3
Day 1	0.328 (0.01)	0.468 (0.01)	0.945 (0.03)	0.469 (0.01)
Day 2	0.416 (0.01)	0.592 (0.01)	1.086 (0.05)	0.592 (0.01)
Day 5	0.508 (0.02)	0.938 (0.02)	1.563 (0.07)	0.945 (0.02)

Table 3.21: Effects of α1(III) CB3 on RWF proliferation.
CB3 at100μg/ml was added to RWF cells (in DMEM supplemented with 2 % serum) and growth after 1, 2 and 5 days was measured using the Methylene Blue assay. The data above represents absorbances at 630nm. Standard errors are shown (brackets). Control values are shown. Negative control - cells grown in DMEM only (0% serum). Positive control – cells grown in DMEM with 10% serum.

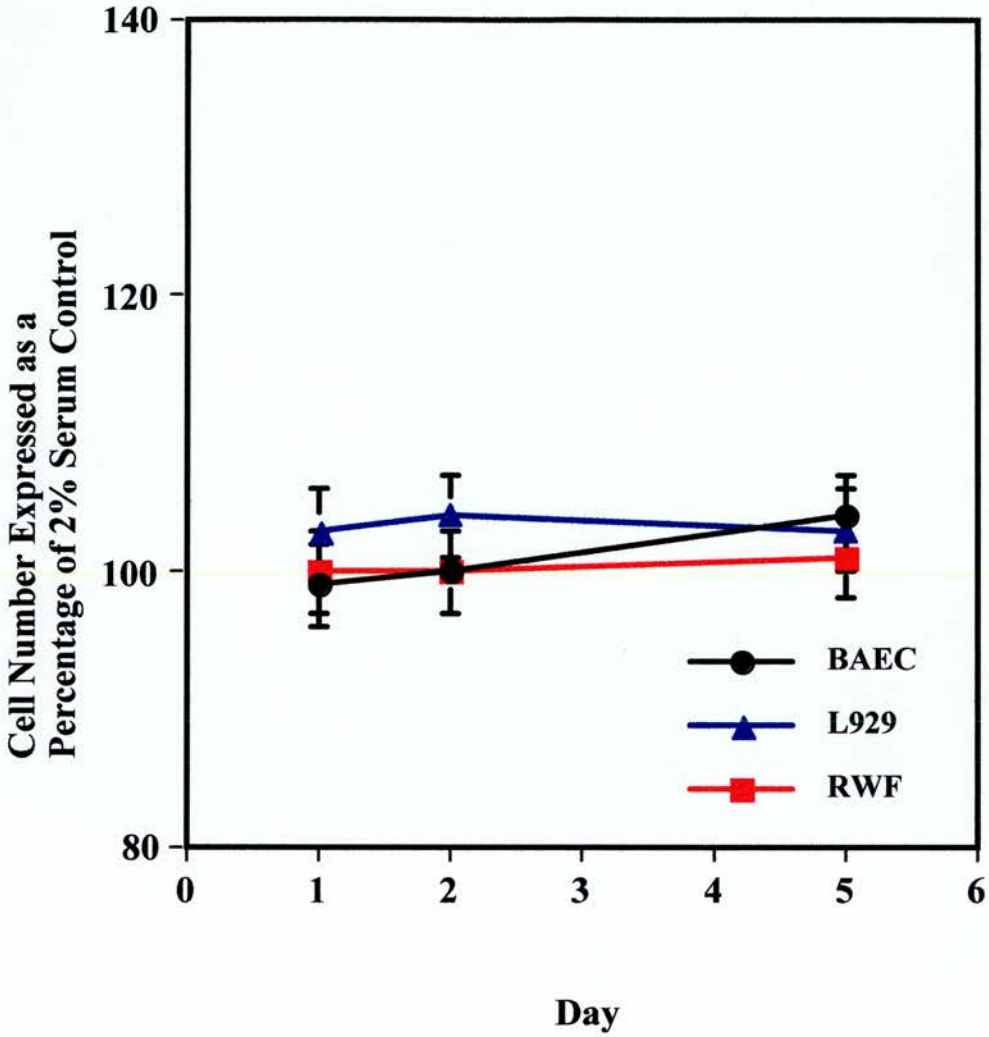

FIGURE 3.17: Effects of α1(III) CB3 on cell proliferation
The cell lines used were BAEC, L929 and RWF. Peptide CB3 was added at a concentration of 100μg/ml and the growth of the three cell types was measured over 5 days. Cells were seeded at a density of 5000 cells/well in 96-microwell plates containing 100μl of DMEM + 2% FCS. Each value represents the mean of 6 wells (Student's t-Test). Standard errors are shown.

BAEC	0% serum	2% serum	10% serum	2% serum +CB5
Day 1	0.441 (0.02)	0.641 (0.01)	1.119 (0.04)	0.666 (0.01)
Day 2	0.561 (0.03)	0.864 (0.02)	1.273 (0.04)	0.891 (0.02)
Day 5	0.874 (0.03)	1.600 (0.03)	1.936 (0.05)	1.631 (0.03)

Table 3.22: Effects of α1(III) CB5 on BAEC proliferation.
CB5 at 100μg/ml was added to cell line BAEC (in DMEM supplemented with 2 % serum) and growth after 1, 2 and 5 days was measured using the Methylene Blue assay. The data above represents absorbances at 630nm. Standard errors are shown (brackets). Control values are shown. Negative control - cells grown in DMEM only (0% serum). Positive control – cells grown in DMEM with 10% serum.

L929	0% serum	2% serum	10% serum	2% serum +CB5
Day 1	0.386 (0.02)	0.617 (0.01)	1.204 (0.03)	0.617 (0.01)
Day 2	0.491 (0.04)	0.784 (0.02)	1.413 (0.05)	0.800 (0.02)
Day 5	0.601 (0.01)	1.419 (0.03)	2.184 (0.06)	1.450 (0.03)

Table 3.23: Effects of α1(III) CB5 on L929 proliferation.
CB5 at 100μg/ml was added to L929 cells (in DMEM supplemented with 2 % serum) and growth after 1, 2 and 5 days was measured using the Methylene Blue assay. The data above represents absorbances at 630nm. Standard errors are shown (brackets). Control values are shown. Negative control - cells grown in DMEM only (0% serum). Positive control – cells grown in DMEM with 10% serum.

RWF	0% serum	2% serum	10% serum	2% serum +CB5
Day 1	0.328 (0.01)	0.584 (0.01)	0.941 (0.03)	0.561 (0.01)
Day 2	0.410 (0.01)	0.621 (0.01)	1.125 (0.04)	0.593 (0.01)
Day 5	0.498 (0.02)	0.944 (0.02)	1.525 (0.06)	0.945 (0.02)

Table 3.24: Effects of α1(III) CB5 on RWF proliferation.
CB5 at 100μg/ml was added to RWF cells (in DMEM supplemented with 2 % serum) and growth after 1, 2 and 5 days was measured using the Methylene Blue assay. The data above represents absorbances at 630nm. Standard errors are shown (brackets). Control values are shown. Negative control - cells grown in DMEM only (0% serum). Positive control – cells grown in DMEM with 10% serum.

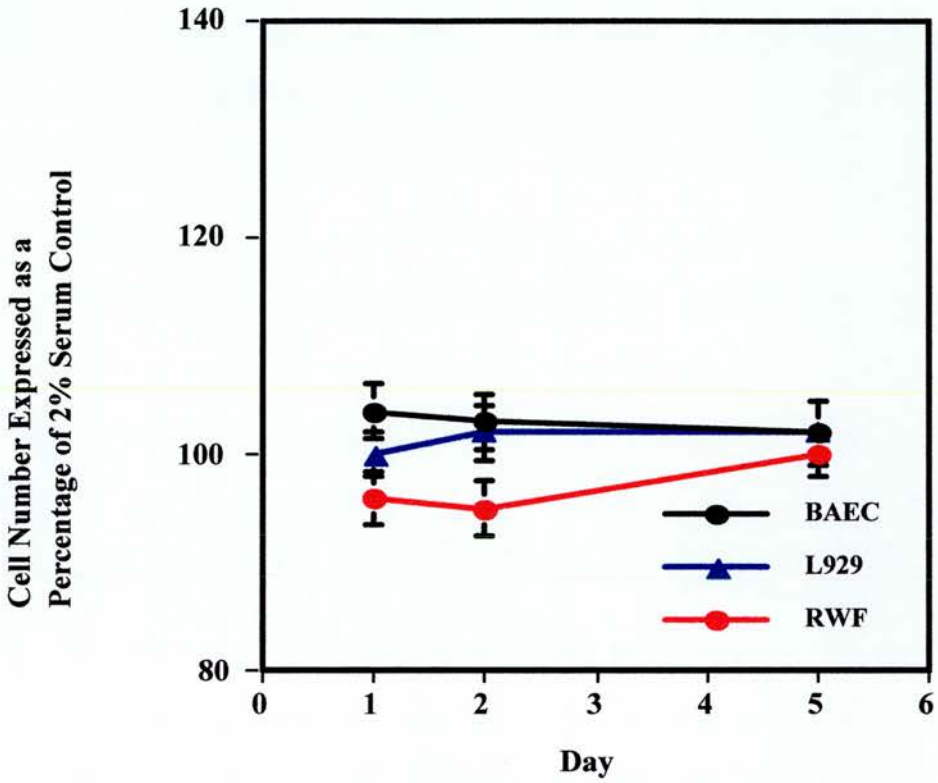

FIGURE 3.18: Effects of α1(III) CB5 on cell proliferation
The cell lines used were BAEC, L929 and RWF. Peptide CB5 was added at a concentration of 100μg/ml and the growth of the three cell types was measured over 5 days. Cells were seeded at a density of 5000 cells/well in 96-microwell plates containing 100μl of DMEM + 2% FCS. Each value represents the mean of 6 wells (Student's t-Test). Standard errors are shown.

Cells grown in DMEM only (negative control) did exhibit a little growth (Tables 3.1-3.24). This could be due to background levels of serum, as cells were grown in the presence of serum before being used in these experiments. This could have been counteracted by starving the cells before starting the assay, however these conditions were found to be deleterious. Perhaps a true serum-free medium where proteins are controlled could be used in the future.

3.7 EFFECTS OF COLLAGEN PEPTIDES ON CELL ATTACHMENT

An inhibitory effect on cell growth in the presence of collagen peptides was frequently seen soon after plating (Day 1). This occurred with both Collagen I and III peptides (Figures 3.7, 3.9, 3.11, 3.13, 3.15). In order to test whether the peptides were preventing attachment of cells to the tissue culture plastic, a cell attachment assay was carried out (Section 2.5.6).

Cell attachment of all three cell lines (BAEC, L929 & RWF) in the presence of peptides α1(I) CB3 and α1(III) CB8 were monitored (Figures 3.19 & 3.20). Cells were grown in the presence of the collagen peptide (concentration-100μg/ml) and DMEM + 2% FCS over a period of 4 hours. Cell number was quantified using the Methylene Blue dye-binding assay (Section 2.5.7; Oliver *et al.*, 1989). Cell attachment/growth was measured after 60, 90, 120, 180 and 240 minutes.

These peptides did not affect the attachment of cells to tissue culture plates, as assessed by comparison with control cells grown only in the presence of DMEM + 2% FCS. Cells grown in the presence of collagen peptide exhibited similar growth curves as cells grown in DMEM + 2% FCS only (Figures 3.19 & 3.20).

It was shown (Figures 3.19 & 3.20) that this inhibition on initial cell growth was not due to the collagen peptides preventing attachment of the cells to tissue culture

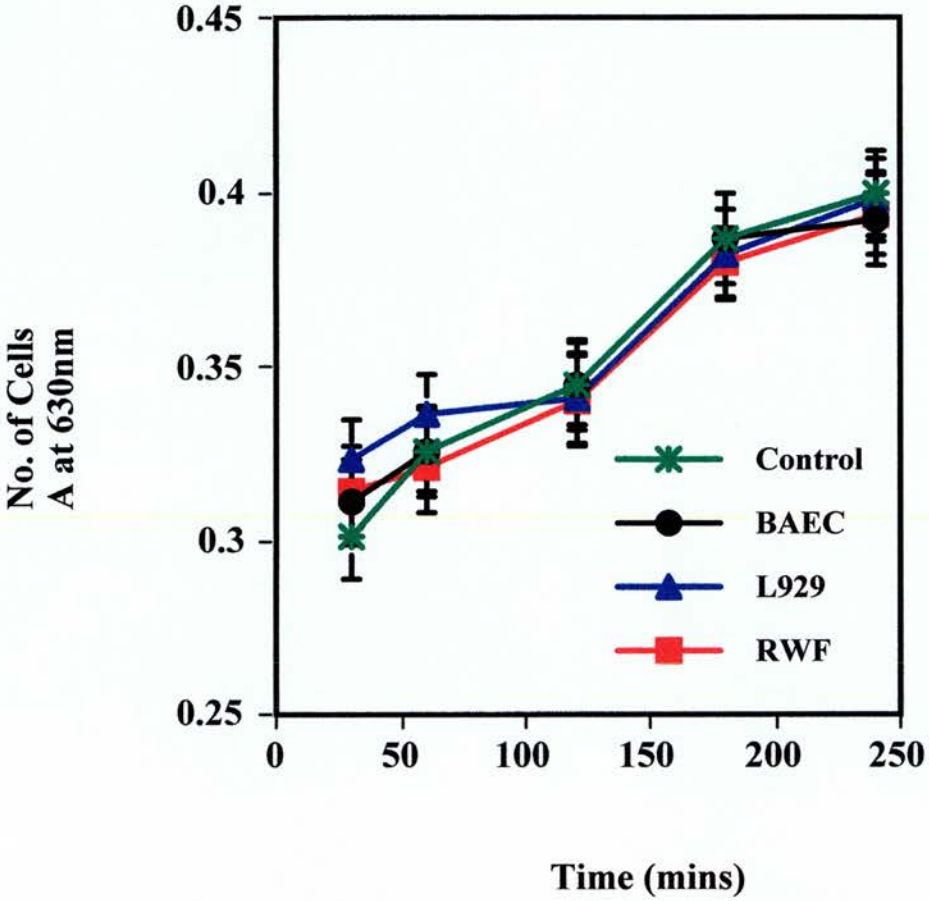

FIGURE 3.19: Cell attachment in the presence of a collagen peptide
L929, BAEC and RWF cell lines were grown in the presence of collagen peptide αI (I) CB3 + DMEM with 2% FCS, at a concentration of 100μg/ml. Control was cells grown in DMEM with 2% FCS in 96-microwell plates and seeded at a density of 5000 cells/well. Each value represents a mean of 6 wells (student's t-test). Standard errors are shown.

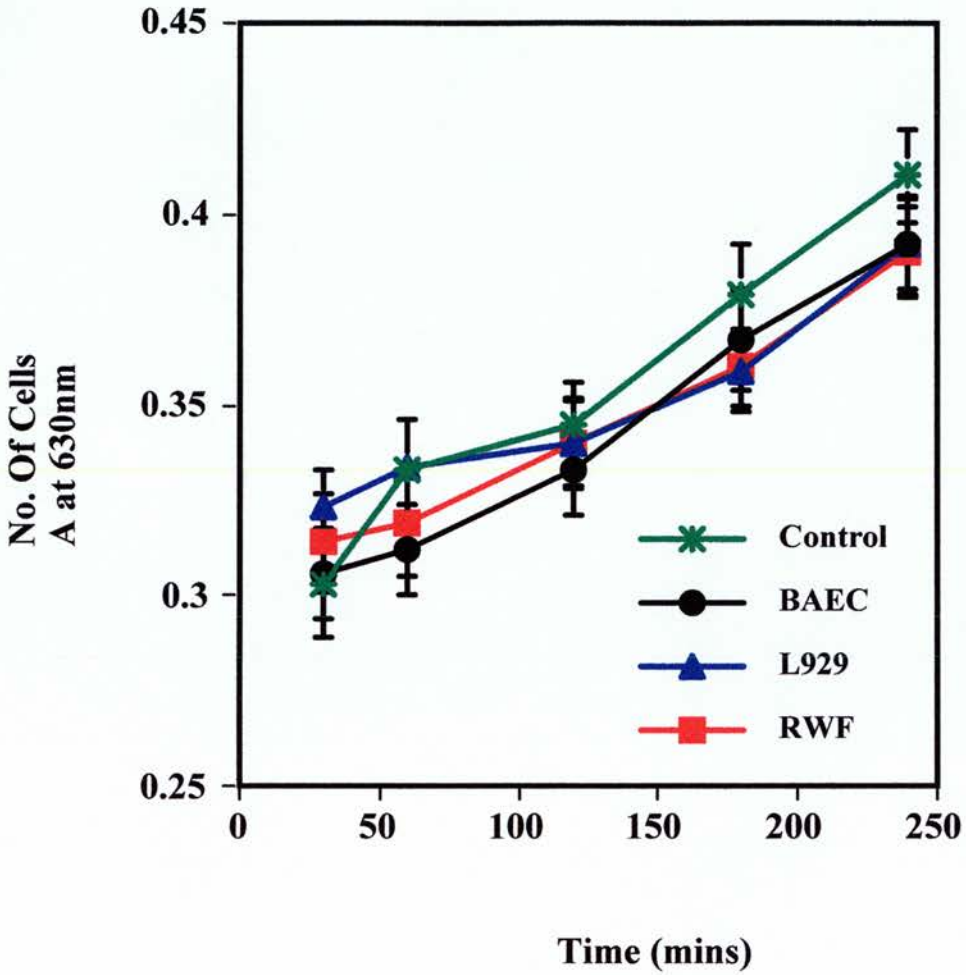

FIGURE 3.20: Cell attachment in the presence of a collagen peptide
L929, BAEC and RWF cell lines were grown in the presence of collagen peptide αI (III) CB8 + DMEM with 2% FCS, at a concentration of 100μg/ml. Control was cells grown in DMEM with 2% FCS in 96-microwell plates and seeded at a density of 5000 cells/well. Each value represents a mean of 6 wells (student's t-test). Standard errors are shown.

plastic. This inhibition may however be due to initial changes in the cellular environment to which the cells eventually adapt i.e. cells may take time to adjust to the addition of the collagen peptide.

3.8 EFFECTS ON CHEMOTAXIS

An important part of wound healing is the migration of cells, in particular fibroblasts, to the wound site. The ability of a test substance to induce chemotaxis depends on the presence of appropriate cell surface receptors (Schiffman *et al.*, 1979). Collagen peptides bind to cell surface receptors in fibroblasts (Grotendorst 1984), this may also be the case for L929 fibroblasts. In addition, the collagen peptides also contain certain sequences, which are involved in the interaction with the cellular receptors (Ruoslahti & Pierschbacher 1987). Since the different peptides react in different ways, some may contain more recognition sequences and therefore can stimulate chemotaxis to a greater extent. With the peptides that stimulate cell proliferation, a similar mechanism of action is thought to occur. Again cell surface receptors and recognition sequences on the collagen molecule maybe involved (Ruoslahti *et al.*, 1994).

Therefore, CNBr collagen peptides were tested for their effect on fibroblast chemotaxis *in vitro*, using the Boyden Chamber technique (Section 2.6.1).

The Boyden Chamber Assay is a simple, rapid assay which allows handling of many samples. The volume of chemotactic factor needed is very low (25µl) as is the cell number needed per chamber (Falk *et al.*, 1989). Briefly, 50µl of 4 x 10^5 L929 cells were placed in the upper compartment of the assay chamber, 25µl of collagen peptide was added to the lower chamber. Cells responding to a concentration gradient of collagen peptide will migrate through a porous membrane filter. Cells reaching the bottom surface of the membrane were fixed, stained and counted. The results of these experiments are shown in Figures 3.21-3.24.

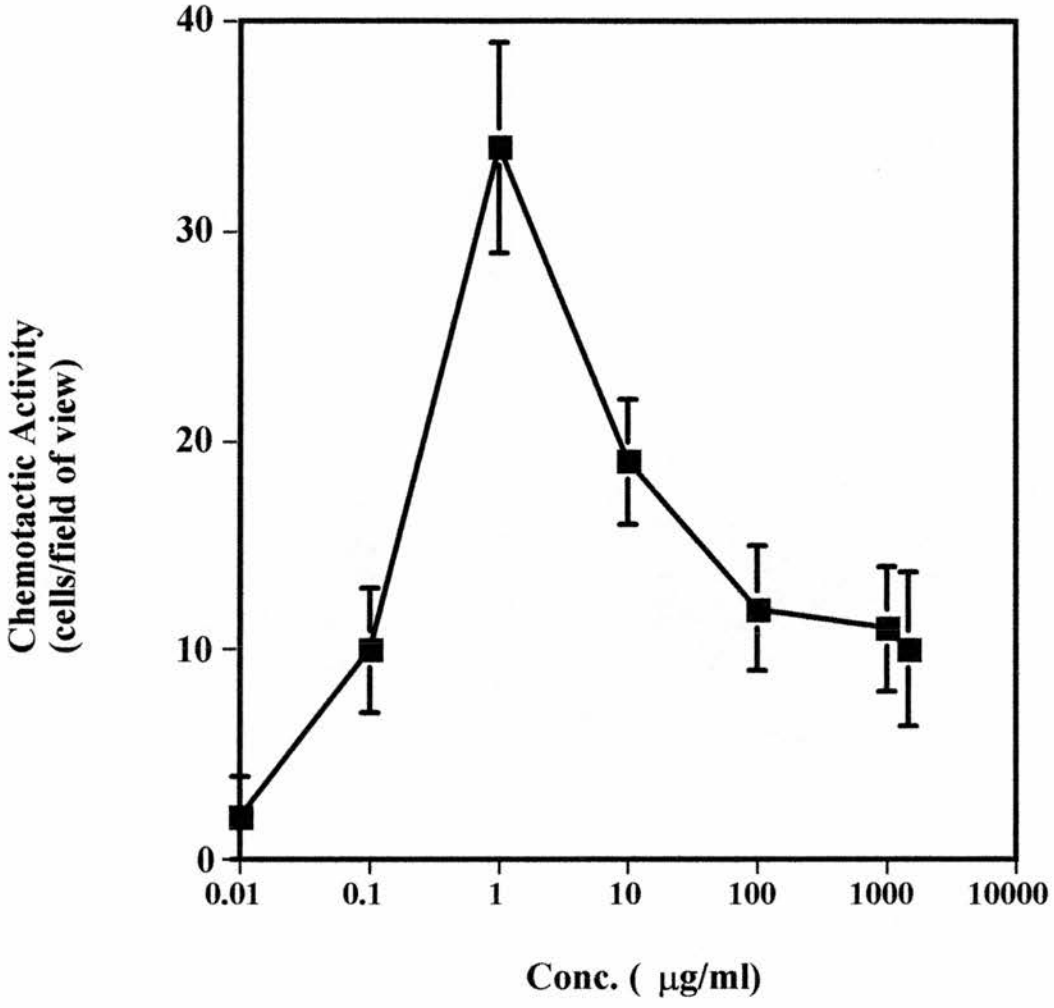

FIGURE 3.21: Effects of α1(I) CB3 on fibroblast chemotaxis.

The migration of L929 fibroblasts in response to collagen peptide fragment α1(I) CB3 was measured. CB3 was added to lower compartments of a Boyden Chamber at the concentrations shown above. The net cell migration from the upper compartment was measured by counting the number of cells per field of view at x40 magnification. Cells which migrated after incubation were fixed to the filter, stained and counted. Points represent the observed chemotactic activity plus or minus standard error for triplicate wells minus the activity of buffer control (55 +/- 1.2).

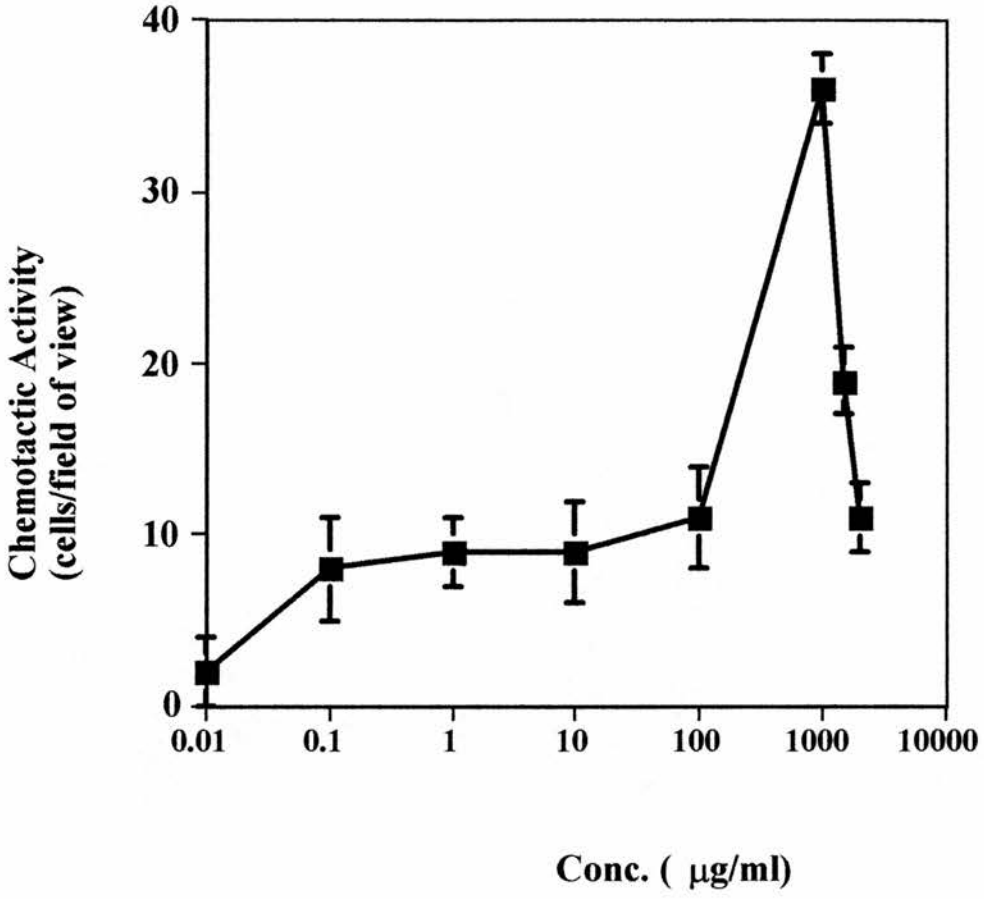

FIGURE 3.22: Effects of α1(I) CB8 on fibroblast chemotaxis.

The migration of L929 fibroblasts in response to collagen peptide fragment α1(I) CB8 was measured. CB8 was added to lower compartments of a Boyden Chamber at the concentrations shown above. The net cell migration from the upper compartment was measured by counting the number of cells per field of view at x40 magnification. Cells which migrated after incubation were fixed to the filter, stained and counted. Points represent the observed chemotactic activity plus or minus standard error for triplicate wells minus the activity of buffer control (56 +/- 1.1).

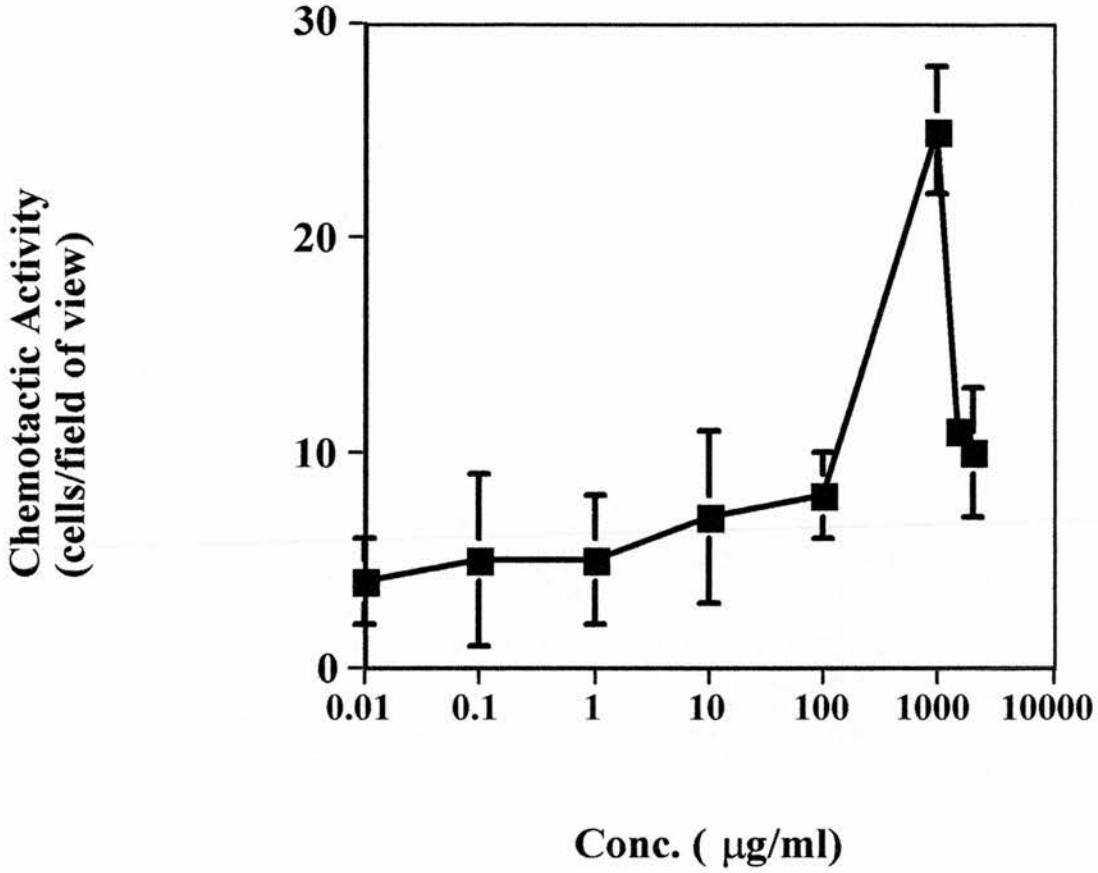

FIGURE 3.23: Effects of α1(III) CB4 on fibroblast chemotaxis.

The migration of L929 fibroblasts in response to collagen peptide fragment α1(III) CB4 was measured. CB4 was added to lower compartments of a Boyden Chamber at the concentrations shown above. The net cell migration from the upper compartment was measured by counting the number of cells per field of view at x40 magnification. Cells which migrated after incubation were fixed to the filter, stained and counted. Points represent the observed chemotactic activity plus or minus standard error for triplicate wells minus the activity of buffer control (57 +/- 1.2).

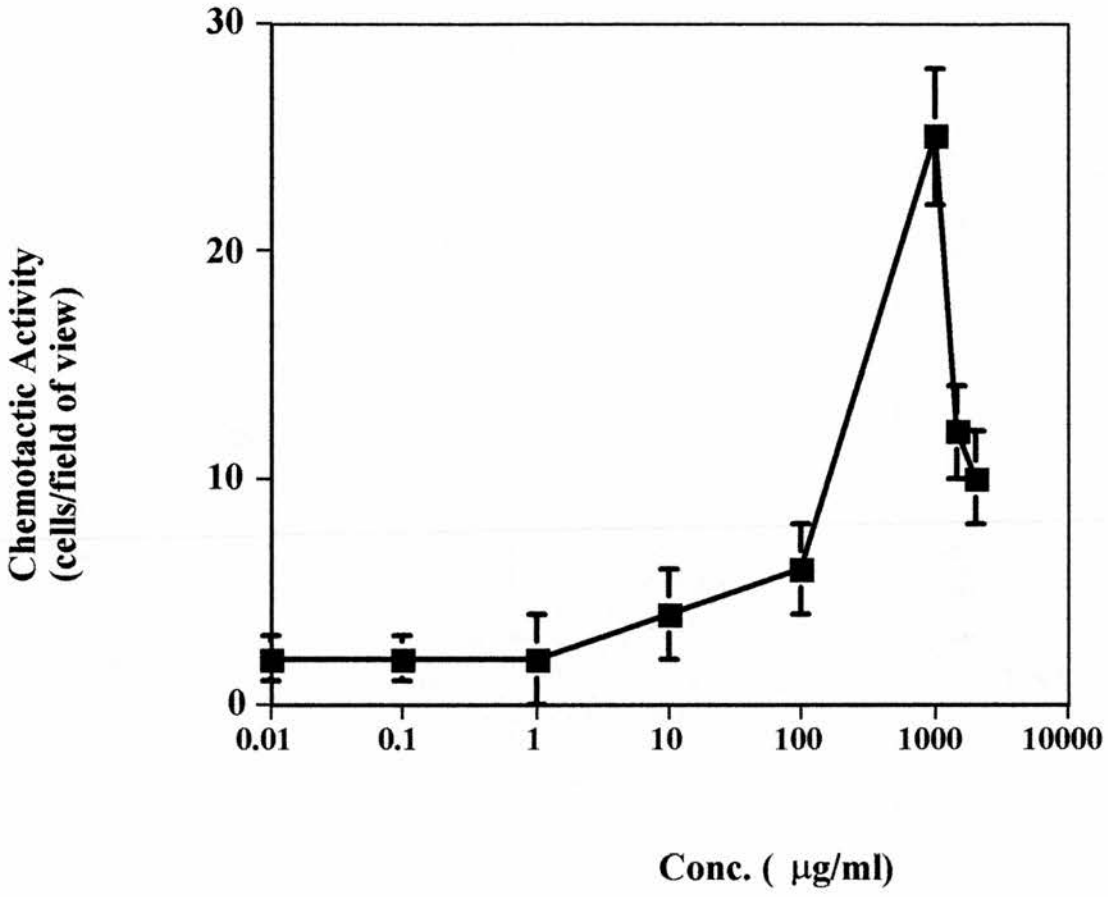

FIGURE 3.24: Effects of α1(III) CB8 on fibroblast chemotaxis.

The migration of L929 fibroblasts in response to collagen peptide fragment α1(III) CB8 was measured. CB8 was added to lower compartments of a Boyden Chamber at the concentrations shown above. The net cell migration from the upper compartment was measured by counting the number of cells per field of view at x40 magnification. Cells which migrated after incubation were fixed to the filter, stained and counted. Points represent the observed chemotactic activity plus or minus standard error for triplicate wells minus the activity of buffer control (56 +/- 1.2).

All of the CNBr fragments (CB3, CB6, CB7 & CB8 from α1(I) and CB3, CB4, CB5 & CB8 from α1(III)) examined from Collagens I and III exhibited chemotactic activity towards fibroblasts. Chemotaxis was always concentration-dependent, producing a bell shaped response curve. Results for the peptides which induced optimal chemotaxis are shown.

Of the Collagen I fragments, α1(I) CB3 and α1(I) CB8 were the most chemotactic, with CB3 showing the greatest effect (Figure 3.21). At 1μg/ml (optimal concentration) chemotaxis was increased by 62% above control levels (random cell migration/movement) with CB3. CB8 increased chemotaxis to a similar degree (Figure 3.22), with 1000μg/ml giving greatest activity.

The CNBr fragments from collagen III, CB4 and CB8, were the most chemotactic. Peptide α1(III) CB4 increased cell chemotaxis by 44%, at an optimal concentration of 1000μg/ml (Figure 3.23). Peptide α1(III) CB8, at a concentration of 1000μg/ml stimulated chemotaxis by 45% (Figure 3.24).

3.9 SUMMARY

The major CNBr peptides from Collagens I and III were isolated and purified in this section. The biological effects of these CNBr-derived peptides were examined. From Collagen I, CB8 stimulated cell proliferation to the greatest extent, however CB4 from Collagen III was found to stimulate cell proliferation at a more potent concentration. These effects were concentration-dependent. An inhibitory effect on cell proliferation was observed, soon after the addition of collagen peptides to the cell lines. This inhibition was not due to the prevention of cell attachment. In the studies on chemotaxis, peptide α1(I) CB3 increased chemotaxis to the greatest degree, at a more potent concentration than observed with the other collagen-derived peptides. These studied on the effects of collagen peptides on chemotaxis and proliferation were investigated further in Chapter 4.

CHAPTER 4:

ISOLATION OF A BIOACTIVE COLLAGEN PEPTIDE

4.1 INTRODUCTION

It has previously been shown that collagen-derived peptides are chemotactic towards a variety of cell types. These peptides generated during wound formation are thought to play an important role in the repair of wounded tissue. In many studies collagen has been degraded by a number of different processes and the batch of peptides generated examined for their biological effects on cell behaviour (Postlethwaite *et al.*, 1978; Riley *et al.*, 1984; Albini & Adelmann-Grill 1985; Laskin *et al.*, 1986; Malone *et al.*, 1991). However, no specific bioactive peptide(s) from collagen has ever been isolated and sequenced.

The aim of this study was to generate a variety of collagen-derived peptides, to be tested for their effects on cell behaviour, in particular chemotaxis (directed cell movement), in order to isolate the peptide(s) which would stimulate cell movement to the greatest extent. Chemotaxis is an important cell behaviour, as it is crucial to wound repair to attract cells which contribute to healing to the wound site. The peptide(s) could then be tested for other biological activities both *in vitro* and *in vivo*, and eventually used to enhance the process of wound healing.

4.2 ENZYMATIC DIGESTION OF COLLAGENS

In order to generate a range of collagen peptides, which could then be tested for their ability to stimulate chemotaxis, Collagen types I, III and V (in their native forms) were digested with a variety of reagents (Section 2.3), namely (a) CNBr (Chapter 3), (b) bacterial collagenase, (c) bacterial collagenase followed by trypsin and finally (d) bacterial collagenase followed by chymotrypsin. After heat-inactivating the enzymes at 95°C for 4hrs: (Levitsky *et al.*, 1994), these crude collagen digests were then tested *in vitro*, in the Boyden Chamber, for their

potential to stimulate chemotaxis (see below).

The concentration of collagen and collagen peptides was determined throughout this study using the Sircol Collagen Assay (see Materials & Methods 2.7.5.3).

Chemotaxis was assayed by using the Boyden Chamber (see Materials & Methods 2.6.1). 50µl of L929 fibroblast cells at a concentration of 4×10^5 cells/ml in DMEM were placed in the top compartment of the chamber. 27µl of the digested collagen fragments in Glycylglycine Buffered Saline (GGBS)+Dulbecco's Modified Eagle's Media (DMEM) was placed in the bottom compartment. The chamber was incubated at 37°C for 2 ½ hours. Fibroblasts which migrated through the PVA filter towards the collagen fragments were fixed in methanol, stained in Diff-Quick and counted. Cells were counted at x40 magnification. Twenty fields of view were counted to give a mean value for number of cells migrated. Buffer only controls (GGBS+DMEM) were used in all experiments and represented background levels of chemotactic activity.

It is important to determine what effect varying the conditions of collagen degradation had upon the chemotactic properties of the peptides produced. For this purpose collagen was digested at a range of enzyme concentrations and as a function of both time and temperature. Peptides recovered from these were then tested for chemotactic activity. The results of an analysis in which Type I collagen was digested with collagenase are shown in Table 4.1. It appears from these data that conditions which produced more extensive degradation (higher enzyme to protein ratio, elevated temperature or longer time) produced peptides with greater chemotactic activity.

Substance Tested	Condition	Maximum Chemotactic Activity [a] (cells/field) enzyme:collagen:ratio (w/w) 1:10	1:100	1:1000
Type I + collagenase	18hrs; 25°C	100.3+/-2.0	65.3+/-1.1	64.0+/-1.4
Type I + collagenase	24hrs; 25°C	112.6+/-1.8	75.8+/-1.7	69.0+/-1.6
Type I + collagenase	18hrs; 37°C	136.2+/-2.2	90.0+/-1.9	72.5+/-1.5
Type I + collagenase	24hrs; 37°C	150.3+/-2.1	121.5+/-2.0	78.7+/-1.4
Buffer Only	n/a	57.6+/-1.4	57.6+/-1.4	57.6+/-1.4
Buffer + collagenase	n/a	92.3+/-1.7	57.4+/-1.5	57.4+/-1.4

a - (mean +/- SEM), n=9

Table 4.1: Collagen I degradation conditions and their effects on chemotaxis
Collagen I (1mg/ml) was degraded by bacterial collagenase at different conditions (temperature, time and enzyme concentration). The chemotactic response of L929 fibroblasts to the collagen peptides generated from each condition was measured using the Boyden Chamber Assay. Buffer used was GGBS + DMEM. Results show the mean of nine samples with standard errors (SEM).

However, enzymes themselves can be chemotactic, as shown by the data in Table 4.1. The most chemotactic effect was observed with Type I collagen degraded with collagenase, with an enzyme to protein ratio of 1:10, incubated at 37°C for 24 hours. Lesser chemotactic effects were seen with Type I collagen degraded with collagenase at enzyme to protein ratios of 1:100 and 1:1000.

The enzymes (collagenase, trypsin and chymotrypsin) were tested alone (without collagen) for their chemotactic potencies. The results of this are shown in Table 4.2. The digestions were carried out for 37°C for 24hrs. At concentrations of 1μg/ml and 10μg/ml the enzymes had no effects on chemotaxis (similar to control values i.e. approx. 57 cells migrated per field of view), however at 100μg/ml the enzymes were found to be chemotactic (Table 4.2). Collagenase and chymotrypsin combined were most chemotactic. Trypsin was least chemotactic. A possible reason for the difference between the enzymes could be that they contain different receptors on their surfaces which only certain cells will recognise and bond to, therefore stimulating the cell to move, as in chemotaxis.

Therefore, in order to reduce the chemotactic effects the enzymes themselves exhibit, only concentrations of enzyme which did not elicit a chemotactic response would be used. From the results in Tables 4.1 and 4.2, the standard conditions for degradation of collagen used would be at 37°C for 24 hours, using an enzyme concentration of 10μg/ml.

Collagens I, III and V were degraded using the standardised conditions previously mentioned. Their degradation products were then tested for chemotactic activity. Results from these experiments are shown in Tables 4.3, 4.4 and 4.5. Native collagens I, III and V were found to be chemotactic as well as the peptides of these collagens produced by digestion with collagenase, CNBr, collagenase followed by

Substance Tested	Maximum Chemotactic Activity (cells/field)[a] conc. of enzyme		
	1μg/ml	10μg/ml	100μg/ml
bacterial collagenase	57.4+/-1.4	57.4+/-1.5	92.3+/-1.7
trypsin	57.2+/-1.6	57.8+/-1.8	66.9+/-1.4
chymotrypsin	58.1+/-1.9	57.9+/-1.7	70.2+/-1.6
collagenase + trypsin	58.2+/-2.0	57.5+/-1.8	78.4+/-1.4
collagenase + chymotrypsin	57.4+/-1.3	57.6+/-1.5	93.6+/-1.8
Buffer only	57.6+/-1.4	57.6+/-1.4	57.6+/-1.4

a - mean +/- SEM, n=9

Table 4.2: The ability of enzymes to stimulate fibroblast chemotaxis
The enzymes named above were examined at three concentrations (1μg/ml, 10μg/ml &100μg/ml) for their ability to induce chemotaxis in L929 fibroblast cells. The Boyden Chamber Assay was used to measure chemotaxis. Buffer used was GGBS + DMEM. Results show the mean of nine samples with standard errors (SEM).

Substance Tested	Maximum Chemotactic Activity (cells/field)[a]
Type I collagen	**83.1+/-1.8**
Type I + collagenase	**121.5+/-2.0**
Type I + trypsin	**58.3+/-1.4**
Type I + chymotrypsin	**58.1+/-1.5**
Type I + CNBr	**72.3+/-1.8**
Type I + collagenase + trypsin	**95.6+/-1.4**
Type I + collagenase + chymotrypsin	**147.6+/-2.2**
Buffer control	**57.6+/-1.4**

a - (mean +/- SEM), n=9

Table 4.3: Degradation of Collagen I and its effect on chemotaxis
Collagen I or its degradation products were incubated for 24hrs at 37°C with different enzymes (enzyme:collagen 1:100 w/w). Collagen degraded by CNBr was also tested for its ability to induce chemotaxis in L929 fibroblasts. The Boyden Chamber Assay was used to measure chemotaxis. Buffer used was GGBS + DMEM. Results show the mean of nine samples with standard errors (SEM).

Substance Tested	Maximum Chemotactic Activity (cells/field)[a]
Type III collagen	**78.2+/-1.5**
Type III + collagenase	**101.0+/-1.8**
Type III+ trypsin	**58.0+/-1.4**
Type III + chymotrypsin	**57.2+/-2.0**
Type III + CNBr	**64.8+/-1.8**
Type III + collagenase + trypsin	**85.2+/-1.4**
Type III+ collagenase + chymotrypsin	**88.4+/-1.6**
Buffer control	**57.6+/-1.4**

a - (mean +/- SEM), n=9

Table 4.4: Degradation of Collagen III and its effect on chemotaxis
Collagen III or its degradation products were incubated for 24hrs at 37°C with different enzymes (enzyme:collagen 1:100 w/w). Collagen degraded by CNBr was also tested for its ability to induce chemotaxis in L929 fibroblasts. The Boyden Chamber Assay was used to measure chemotaxis. Buffer used was GGBS + DMEM. Results show the mean of nine samples with standard errors (SEM).

Substance Tested	Maximum Chemotactic Activity (cells/field)[a]
Type V collagen	**69.3+/-1.4**
Type V + collagenase	**76.5+/-1.6**
Type V + trypsin	**57.8+/-1.5**
Type V + chymotrypsin	**65.4+/-2.0**
Type V + CNBr	**78.0+/-1.8**
Type V+ collagenase + trypsin	**82.6+/-1.9**
Type V + collagenase + chymotrypsin	**57.6+/-1.4**
Buffer control	**57.6+/-1.4**

a - (mean +/- SEM), n=9

Table 4.5: Degradation of Collagen V and its effect on chemotaxis
Collagen V or its degradation products were incubated for 24hrs at 37°C with different enzymes (enzyme:collagen 1:100 w/w). Collagen degraded by CNBr was also tested for its ability to induce chemotaxis in L929 fibroblasts. The Boyden Chamber Assay was used to measure chemotaxis. Buffer used was GGBS + DMEM. Results show the mean of nine samples with standard errors (SEM).

trypsin and finally collagenase followed by chymotrypsin. Table 4.3 shows the results of Type I collagen degradation. The most chemotactic was collagen digested with collagenase and chymotrypsin. This was three times the control level (147.6 compared to 57.6). Type I collagen digested with collagenase followed by trypsin, and also Type I digested with collagenase followed by chymotrypsin were not chemotactic.

From the results in Tables 4.3, 4.4 and 4.5 the optimal conditions for producing maximum chemotactic activity were as follows. Table 4.4 shows the results of Type III collagen digestion. The digestion which produced the greatest chemotactic effect was with collagen digested with collagenase. Table 4.5 shows the results of Type V collagen digestion. The digestion which produced the greatest chemotactic effect was with Type V digested with collagenase and then trypsin.

In Figure 4.1 the concentration-dependence of the peptides which displayed maximal chemotactic behaviour for each collagen type is shown. With Collagens I and III, optimum chemotaxis occurred at a concentration of 100μg/ml. With Type V, 10μg/ml produced optimal chemotaxis. The digest which displayed the most pronounced chemotactic behaviour was that obtained by digestion of collagen I molecules with both collagenase and chymotrypsin. Chemotaxis was increased by almost three times compared to the control sample (GGBS + DMEM).

As Collagen I degradation products were the most chemotactic, future experiments will be focused on Collagen I.

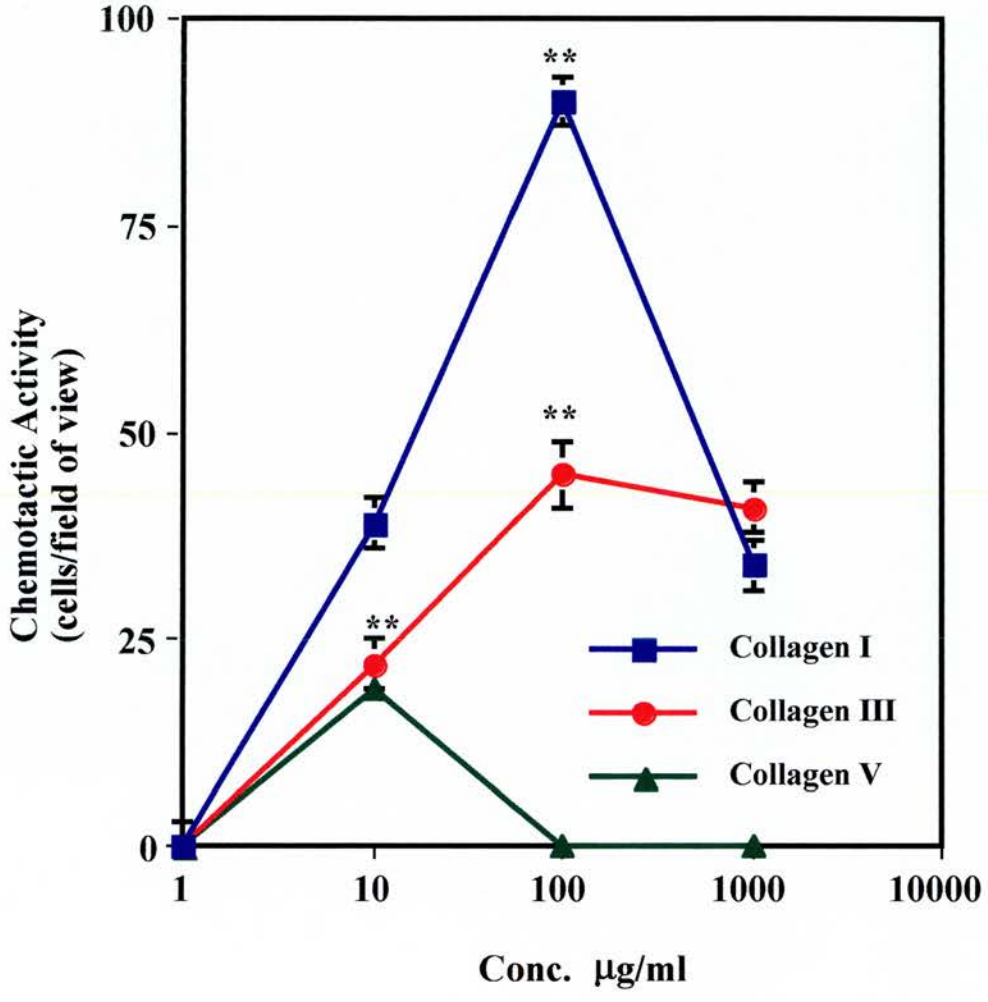

FIGURE 4.1: Chemotactic response of L929 fibroblasts to Type I, III and V collagen peptides

Collagen types I, III and V (1mg/ml) were degraded by a variety of methods in order to produce chemotactic peptides. The degradation methods resulting in maximal chemotactic behaviour for each collagen type were as follows: Type I - collagenase + chymotrypsin, Type III - collagenase, Type V - collagenase + trypsin. Chemotaxis was carried out in the Boyden Chamber Assay. Points represent the observed chemotactic activity +/- SEM minus the activity of buffer (DMEM + GGBS), 57.6+/- 1.4. $p^{*}<0.01$ (Student's t-Test), n=9.

4.3 ISOLATION AND SEPARATION OF COLLAGEN PEPTIDES

Since collagen I digested with collagenase/chymotrypsin (Section 4.2) produced the most chemotactic samples the next step was to isolate the most bioactive collagen peptide(s) from this preparation. To this end the above digest was fractionated using reverse-phase HPLC (Figure 4.2). Collagen samples were dissolved in 0.1% (v/v) trifluoroacetic acid, filtered and then injected onto a C18 reverse-phase column. The column was eluted with a 120ml gradient of 0-40% acetonitrile, delivered at a flow rate of 1ml/min. Fractions were collected every 10 minutes.

4.4 IDENTIFICATION OF THE ACTIVE PEPTIDE

Each fraction obtained from the HPLC separation was freeze-dried, resuspended in GGBS/DMEM and then individually tested in the Boyden chamber for chemotactic activity. All of the fractions contained some activity, however fractions separated between 30-40 min (Figure 4.3, fraction 4: F4) after HPLC showed the most chemotactic activity. All of these separated fractions contained some chemotactic activity. Each bar represented chemotactic activity minus buffer control (GGBS/DMEM), which was 57.2 in this case. Fraction 4 stimulated chemotaxis by almost 100% (a chemotactic activity of 55), compared to control values. After examination of the HPLC profile (figure 4.2), it was decided to run fraction 4 (F4) again through the C18 column and collect the largest peak (* in figure 4.2). The HPLC profile of this peak is shown in Figure 4.4. Its purity was found to be approximately 95%. The peak was then freeze-dried and assayed for its chemotactic activity in the Boyden Chamber Assay. These results are shown in Figure 4.5. The isolated peak was found to stimulate L929 fibroblast chemotaxis at an optimal concentration of 100ng/ml (Fig 4.5). This is the same level of potency as the isolated ten minute fraction (F4), therefore this peak was responsible for the

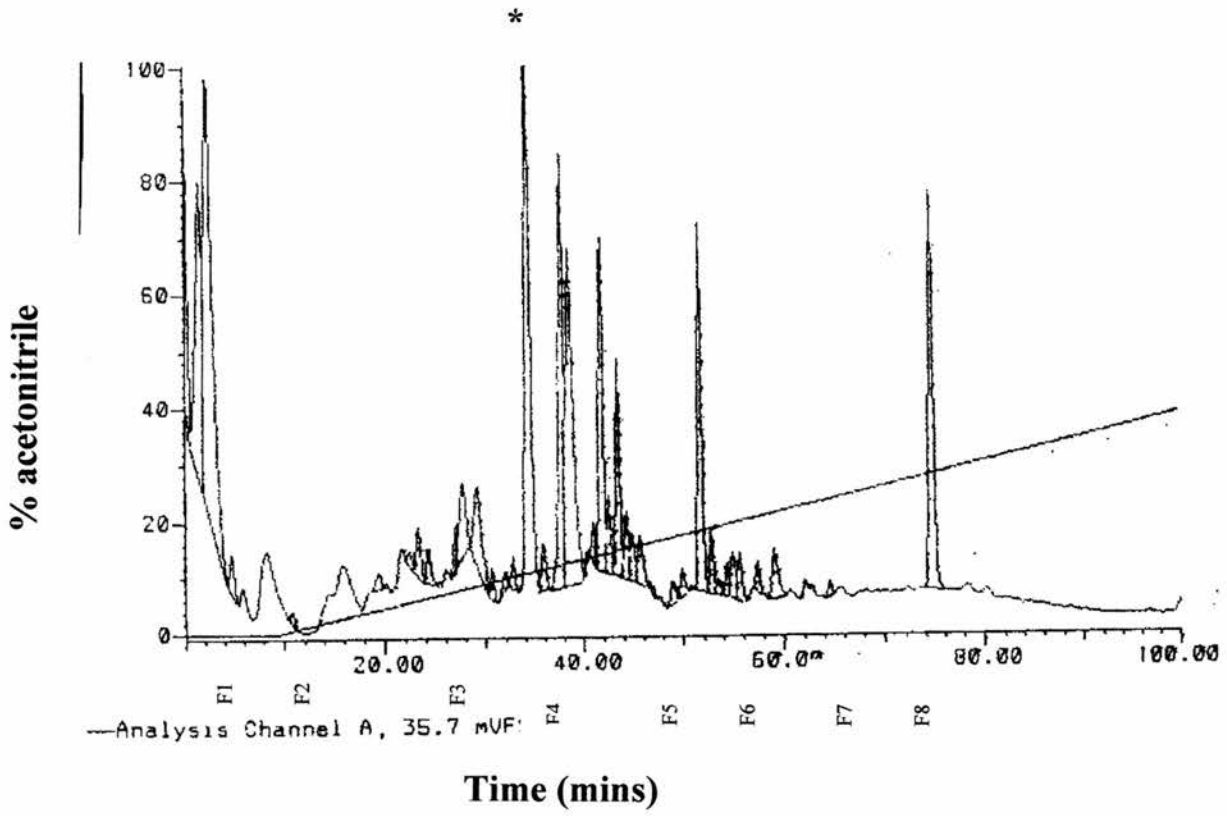

Figure 4.2: Separation of collagen peptides by reverse-phase HPLC
Collagen I chains digested with collagenase and chymotrypsin were separated by reverse-phase chromatography (C18 Dynamax column) into fractions (F1-F8). A 0-40% acetonitrile gradient in 0.1% trifluroacetic acid, at a flow rate of 1ml/min was delivered. Absorbances were measured at 214nm. The most chemotactic fragment was collected between 30-40mins (F4). The peak indicated by * was the fraction thought responsible for this activity.

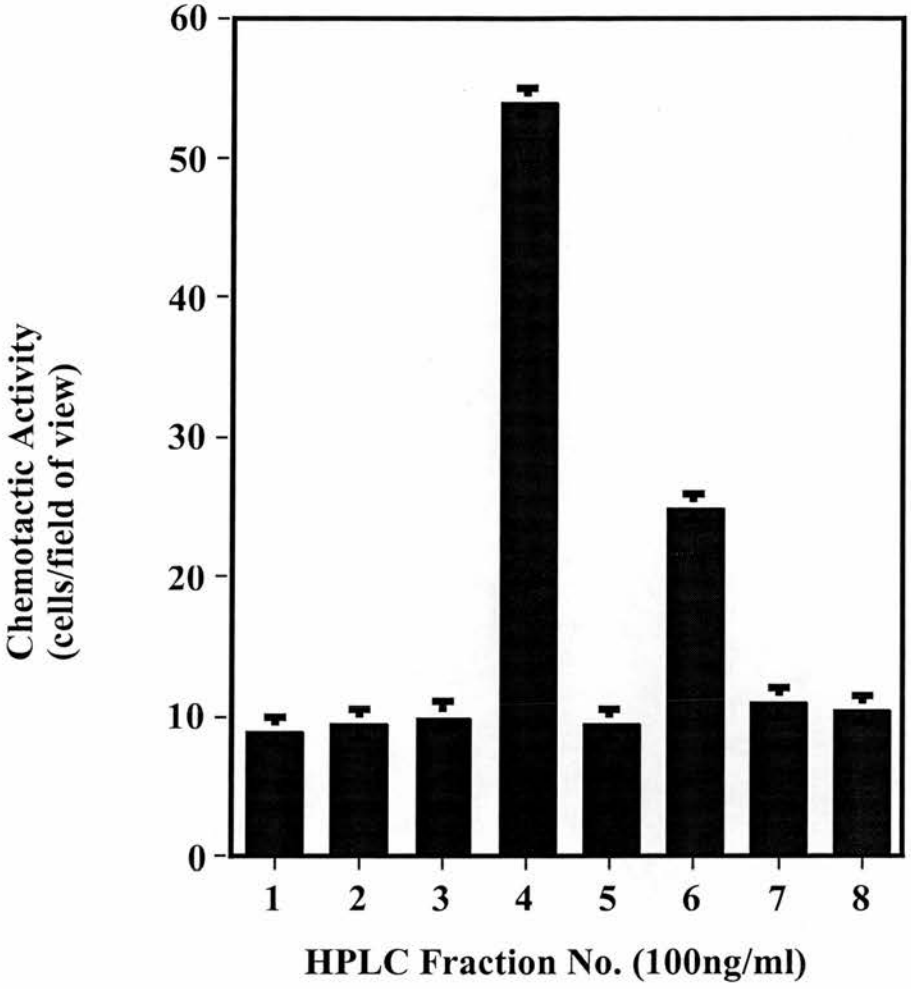

FIGURE 4.3: Chemotactic effect of collagen fractions on L929 fibroblasts
Collagen I digested by collagenase and chymotrypsin was fractionated by reverse-phase HPLC. The fractions collected at 10 minute intervals were tested individually for their effects on chemotaxis in the Boyden Chamber Assay. Bars represents the observed chemotactic activity +/- SEM minus the activity of buffer (GGBS+DMEM) control (57.2+/-1.1). n=9.

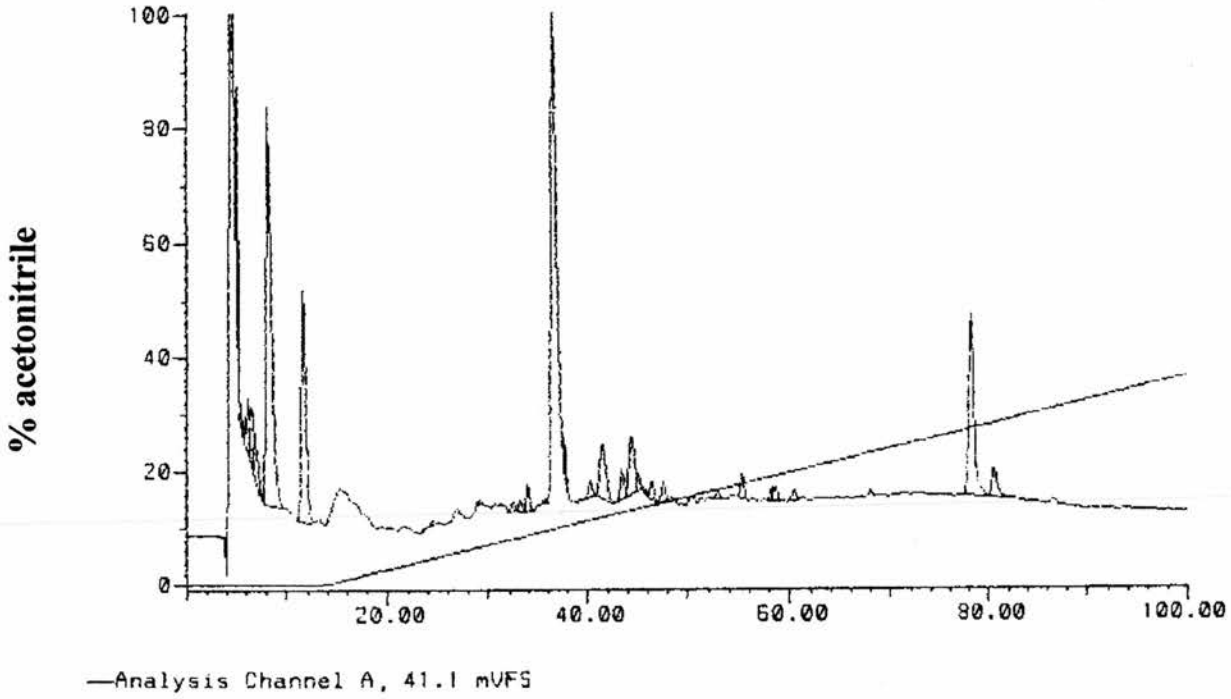

Figure 4.4: HPLC profile of the most chemotactic collagen peptide
Collagen I was digested with collagenase and chymotrypsin. The peptides generated were tested for chemotactic activity. The most chemotactic peptide was isolated and its purity checked by reverse-phase HPLC. The freeze-dried peptide was dissolved in 0.1% trifluroacetic acid, filtered and injected onto a C18 reverse-phase column. The column was eluted with a gradient of 0-40% acetonitrile at a flow rate of 1ml/min. Absorbances were measured at 214nm.

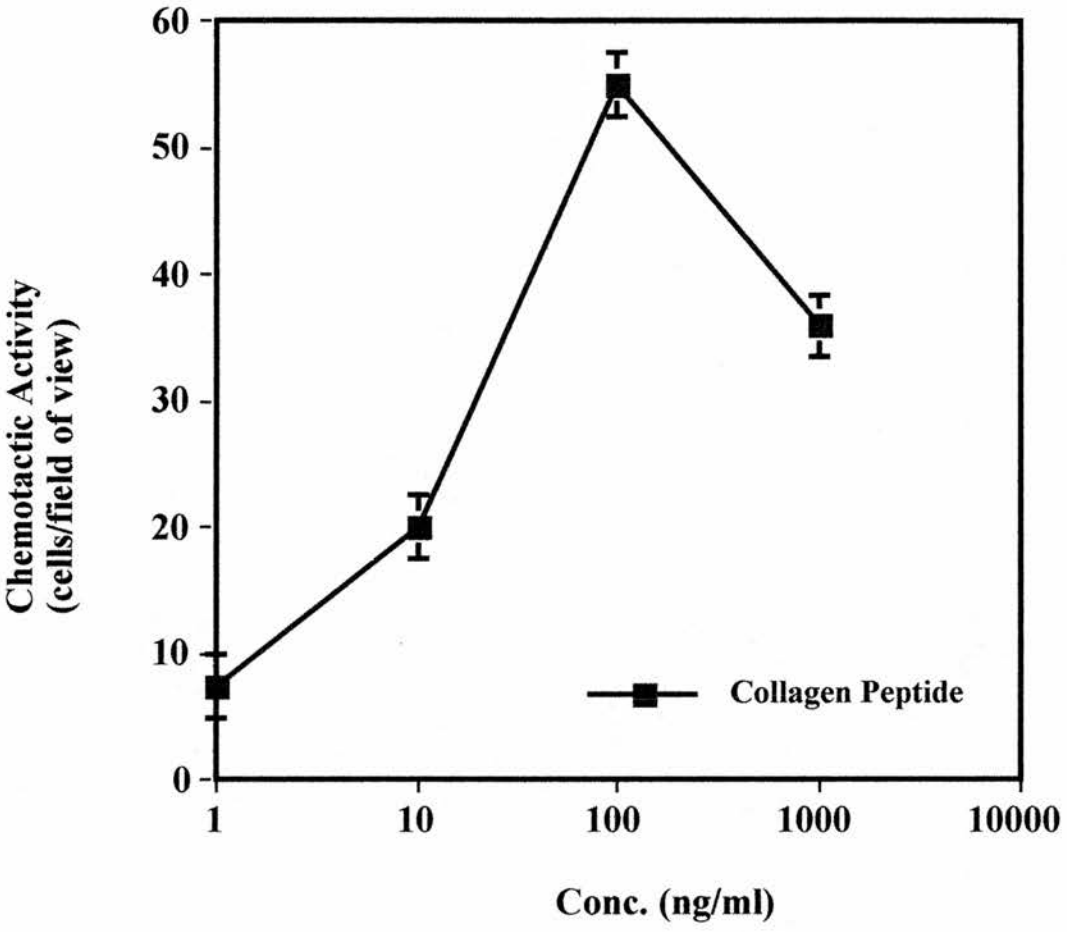

FIGURE 4.5: Chemotactic activity of isolated collagen peptide
The collagen I fraction isolated after enzymatic degradation and HPLC purification (* in Fig 4.2) was tested over a range of concentrations for its ability to induce L929 fibroblast chemotaxis. Chemotaxis was carried out in the Boyden Chamber Assay. Cells were seeded at a density of 5000 cells/well and grown in microwell plates containing 100µl of DMEM + 2% FCS. Standard errors are shown.

chemotactic activity of this fraction.

4.5 *IN VIVO* EFFECTS OF THE ACTIVE PEPTIDE

PVA (polyvinyl alcohol) implants have been used successfully to study wound healing in experimental models (Cooney *et al.*, 1997; Shah *et al.*, 1999). PVA sponges can be used to measure cellular infiltration as they act as a stable dead space into which cells can migrate. The PVA sponge animal model was used in this project to analyse the chemotactic effects of collagen peptides *in vivo* (Section 2.7). Sponges were subcutaneously implanted into male Wistar rats and after three days were injected with 100µl of the crude digest of peptides (0.1mg/ml) derived from collagenase and chymotrypsin degradation of Collagen I. Seven and ten days post-implantation the sponges were removed and analysed both histologically and biochemically (i.e. quantitation of total DNA, protein and collagen). It was the ratios of collagen, DNA and protein in the experimental PVA sponges (injected with collagen peptide) compared to control PVA sponges (PBS buffer only) which was measured.

The ratios of collagen, DNA and protein in the experimental sponges were greater (>1) than in control sponges, 7 days post-implantation (Figure 4.6).These increases were even greater after 10 days. The collagen I digest, from the previous section, at a concentration of 0.1 mg/ml, was found to have significant stimulatory activity on DNA content of tissue after 10 days compared to control sponges (Figure 4.6).

For histological analysis, sections (5 micron) were cut from PVA sponges which had been fixed in formalin, embedded in paraffin and stained with haematoxylin and eosin. Each sponge was assessed for granulation tissue content by examination of

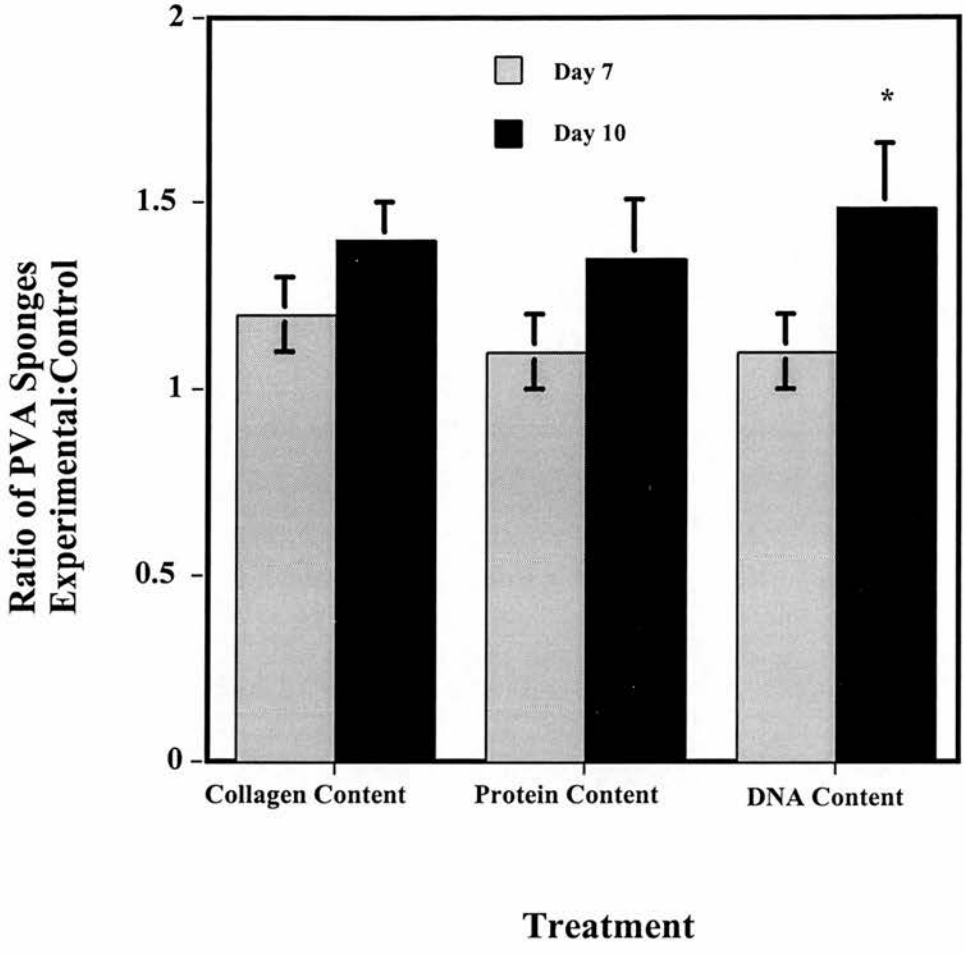

FIGURE 4.6: Biochemical analysis of PVA sponges injected with collagen I peptide(s)

Protein, DNA and collagen contents were measured in PVA sponges injected with 100μl of 0.1mg/ml of collagen peptides (derived after degradation with collagenase and chymotrypsin). Sponges were removed from rats and analysed 7 and 10 days post injection. Results are expressed as a ratio of experimental (sponge + collagen peptide) to control sponge (PBS only). Standard errors are shown, n=10. *$p<0.05$ (Student's t-Test).

these stained sections. Granulation tissue is composed of fibroblasts and inflammatory cells (macrophages/monocytes).

The granulation tissue was assessed microscopically (i.e. 'eyeballing') by relating the content of the sponges containing collagen peptide to those containing PBS control. From Figure 4.7, where a= PVA sponge, b= fibrin (thin ribbons) and c= granulation tissue (cellular material), it could be seen that the digest had an overall stimulatory effect at 0.1 mg/ml after 10 days post injection (Figure 4.7) as seen by the formation of granulation tissue. More fibrin and fibroblasts/inflammatory cells can be seen in the collagen peptide sponge (B).

4.6 AMINO ACID SEQUENCE OF PEPTIDE

The amino acid sequence of the chemotactic peptide, isolated by HPLC (Fig 4.2) from collagen I (digested with collagenase and chymotrypsin) was determined using an Applied Biosystems microsequencer (kindly carried out by Dr A Cronshaw). The peptide (Figure 4.8) was found to be 22 amino acids in length and corresponded (by reference to Swiss-Prot data base)to the α2 chain of collagen I (residues 25-46). The peptide was isolated from porcine collagen I. This peptide sequence was found to be identical with the corresponding sequence in bovine collagen I. However, the porcine collagen sequence varies by only one amino acid from the human sequence (residue 36; Thr → Hyp) and differs by three amino acids from that of rat (Fig 4.8; Barnes *et al.*, 1982).

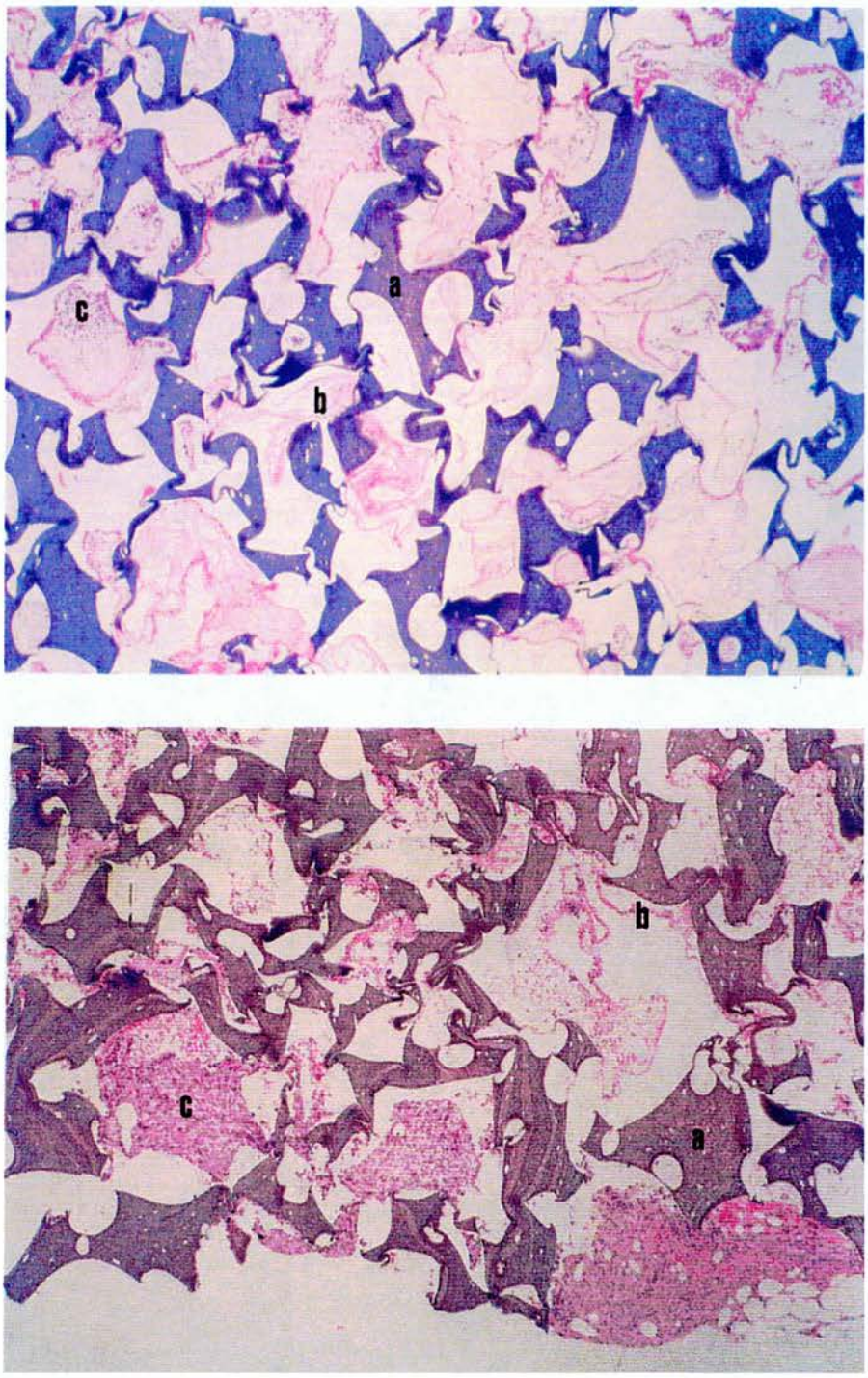

Figure 4.7: PVA sponge histology
PVA sponges were assessed for granulation tissue content in Haematoxylin and Eosin stained sections (magnification x40).
(A) control sponge (PBS only) – little cellular invasion
(B) sponge containing collagen I peptides (0.1mg/ml), removed after 10 days post-implantation – moderate cellular invasion.
a=PVA sponge b=fibrin c=granulation tissue

25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46

(i) Gly-Pro-Hyp-**Gly**-Glu-Hyp-**Gly**-Glu-Hyp-**Gly**-Gln-Hyp-**Gly**-Pro-Ala-**Gly**-Ala-Arg-**Gly**-Pro-Ala-**Gly**

(ii)Gly-Pro-Hyp-**Gly**-Glu-Hyp-**Gly**-Glu-Hyp-**Gly**-Gln-Thr-**Gly**-Pro-Ala-**Gly**-Ala-Arg-**Gly**-Pro-Ala-**Gly**

(iii) Gly-Pro-Hyp-**Gly**-Glu-Hyp-**Gly**-Glu-Hyp-**Gly**-Gln-Hyp-**Gly**-Pro-Ala-**Gly**-Ala-Arg-**Gly**-Pro-Ala-**Gly**

(iv) Gly-Pro-Ala-**Gly**-Glu-Hyp-**Gly**-Glu-Hyp-**Gly**-Gln-Hyp-**Gly**-Pro-Ala-**Gly**-Pro-Arg-**Gly**-Pro-Hyp-**Gly**

Figure 4.8: Species comparison of the amino acid sequence of the isolated collagen peptide
A collagen peptide from pig skin (i) obtained by enzymatic digestion and HPLC purification was shown to be chemotactic for fibroblasts. It was found to be 22 amino acids in length and corresponded to the α2 chain of Collagen I (residues 25-46). The sequence is identical to that of bovine collagen (iii). The same region in human (ii) and rat (iv) differ by one and three amino acids respectively.

4.7 SUMMARY

In this section, peptides derived from Collagen Types I, III and V were generated and their chemotactic potencies determined. It was found that Collagen I digested sequentially with collagenase and chymotrypsin produced peptides with greatest chemotactic effects. The most bioactive peptide from this digest was isolated and its sequence determined. The peptide was then synthesised on a larger scale and its biological activities examined further (Chapter 5).

CHAPTER 5:

BIOLOGICAL EFFECTS OF A SYNTHETIC COLLAGEN PEPTIDE

5.1 INTRODUCTION

Collagen peptides have been shown to be chemotactic towards a variety of cell types. In Chapter 4 an HPLC-purified peptide isolated from Collagen I after sequential enzymatic digestion with bacterial collagenase and chymotrypsin was found to stimulate fibroblast cell migration by 100%. The 22-amino acid sequence of the peptide was found to be from the α2 chain of Collagen I. It was decided that the peptide would be synthesised chemically so that it could then be tested for its biological activities in a variety of systems. In order to localise the chemotactic domain of this 22-amino acid collagen peptide, a variety of synthetic collagen peptides containing amino acid sequences from within this larger peptide were also made and then tested for their chemotactic potential.

Cell migration is essential for the formation of granulation tissue (Chen & Abatangelo, 1999), chemotaxis is directional cell migration. Therefore it is crucial to wound repair to attract cells, that contribute to healing, to the wound site.

5.2 LARGE SCALE PURIFICATION OF PEPTIDE

Thirty milligrams of the 22-amino acid peptide were synthesised by Edinburgh University Chemistry Department using a fully automated Biosystems 430A peptide synthesiser. The FMC/^{t}Bu (9-fluorenlymethoxycarbonyl/tertiary butyl) based method of peptide synthesis was used (Carpino & Han, 1972).

The peptide underwent laser desorption mass spectral analysis to confirm its molecular mass and also amino acid sequencing to confirm that it had the correct sequence.

5.3 HPLC ANALYSIS OF PEPTIDE

The peptide was chromatographed on a C8 reverse-phase column (Aquapore) with a gradient of 90% (H_2O/0.1% TFA) to 90% (aceteonitrile/0.1% TFA) and a flow rate of 1ml/min (by Edinburgh University Chemistry Dept.) and shown to be 95% pure (Figure 5.1).

5.4 CHEMOTACTIC EFFECTS

This peptide was tested for its ability to stimulate chemotaxis with fibroblasts and endothelial cells. Chemotaxis was carried out in the Boyden Chamber Assay as before (see Section 2.6). Briefly, 50µl of L929 fibroblast cells at a concentration of 4 x 10^5 cells/ml in DMEM were placed in the top compartment of the chamber. 25µl of digested collagen fragments and the synthetic peptide (in GGBS+DMEM) were placed in the bottom compartment. The synthesised peptide was chemotactic towards L929 fibroblasts (Figure 5.2) to a similar degree as the collagen-derived sample (see Section 4.4) of the peptide, except that the collagen-derived peptide was slightly more potent. A reason for this slight difference could be that the collagen-derived peptide had some impurities in it, which could also be chemotactic. The synthetic peptide had less impurities (95% pure as stated in Section 5.3). The similar activity of both the synthetic and collagen-derived peptide samples suggests however that the observed chemotaxis is largely due to the amino acid sequence of the collagen and not to some unknown impurities.

The peptide was also tested against other fibroblast cells, in order to examine its ability to stimulate chemotaxis in different cell lines. The L929 and 3T3 fibroblasts are continuous cell lines (cells modified in order to produce a continuously growing cell line), whereas RWF are primary cell lines (cells recently isolated from tissue or organs and have a limited lifespan). Primary cells are more likely to reflect the

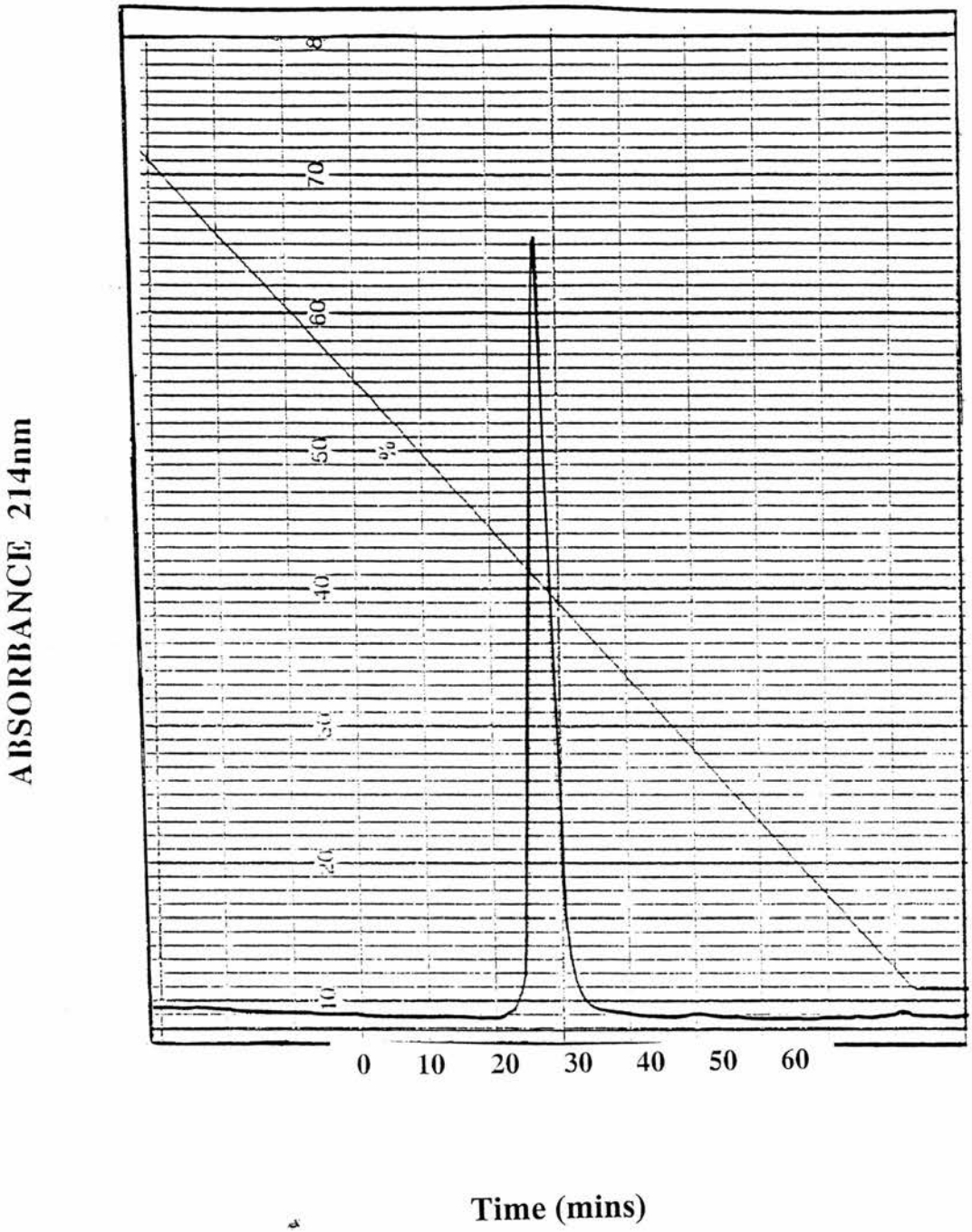

Figure 5.1 : HPLC profile of the synthetic collagen peptide
Purity of the synthesised 22-amino acid peptide was determined by HPLC analysis [Column: C8 Aquapore; Gradient: 90% (H_2O/0.1% TFA) → 90% (acetonitrile/0.1%TFA), flow rate 1ml/min].

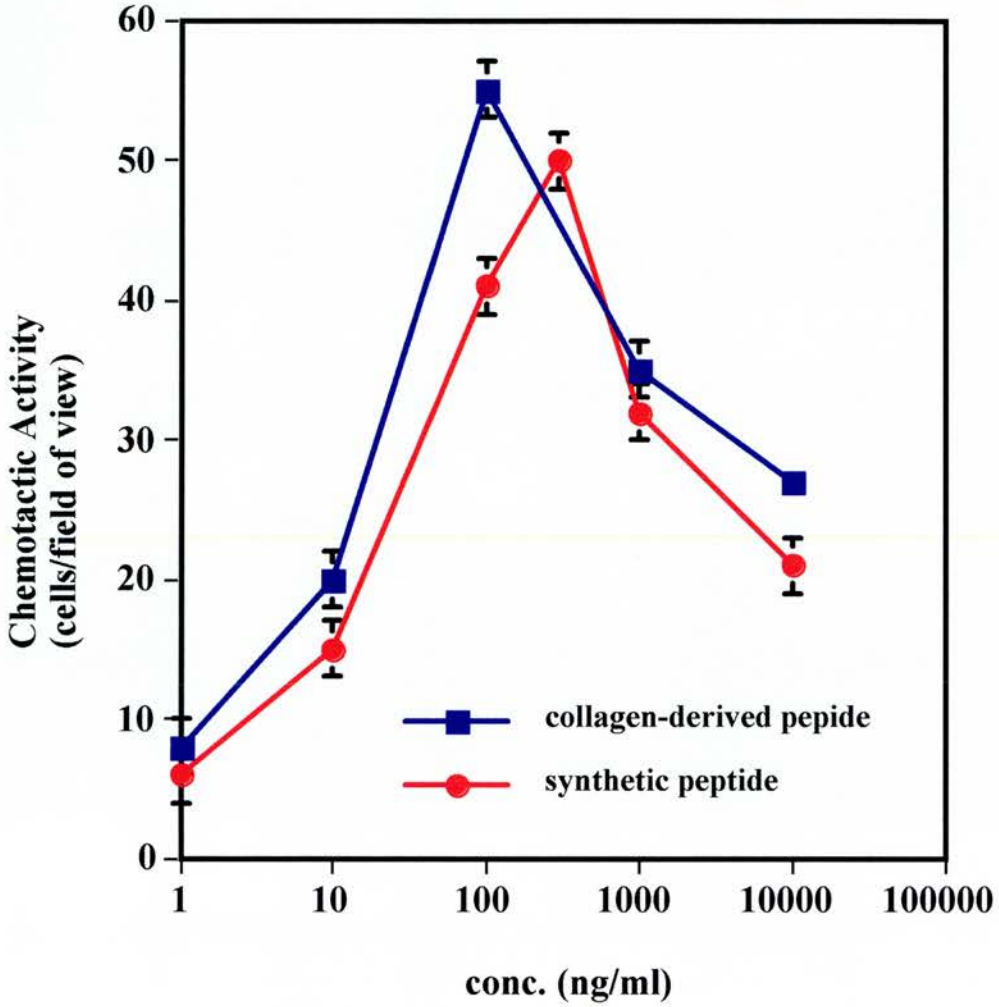

FIGURE 5.2: Effects of both collagen-derived and synthetic collagen peptides on fibroblast chemotaxis

The fibroblast cell line examined was L929. A collagen-derived peptide was compared with a synthetic collagen peptide for their ability to stimulate chemotaxis. The peptides were added to lower compartments of a Boyden Chamber at the concentrations shown above. The net cell migration from the upper compartment was measured by counting the number of cells per field of view. Points represent the observed chemotactic activity +/- SEM for triplicate wells minus the activity of buffer control (56+/- 1.3).

true activity and functions that they display in their natural environment (Morgan & Darling 1993). Hence, it was important to examine the effect of the peptide on these cell lines.

RWF and 3T3 cells were shown to be chemotactic to a similar degree as the L929 fibroblast cells (Figure 5.3).

The peptide was not significantly chemotactic towards endothelial cells (Figure 5.4), even though a bell-shaped chemotaxis curve was produced. This data suggests that endothelial cells do not have cell surface receptors which recognise and bind to the collagen peptide, whereas fibroblasts cells appear to have receptors which are recognised.

The collagen peptide stimulated chemotaxis in a variety of fibroblast cell types, this suggests that fibroblasts have cell surface receptors which bind a region of this collagen peptide.

5.5 "CHECKERBOARD ASSAY" FOR VERIFICATION OF DIRECTIONAL MIGRATION

To determine whether the cell migration was directional in nature (chemotactic), a "checkerboard" analysis was performed, following the method of Zigmond and Hirsch (1973). As chemoattractants can also increase cell movement in a random nature (chemokinesis), this method was used in an attempt to distinguish between these two kinds of cell migration.

The checkerboard was set up essentially as described for the Boyden Chamber Assay. The cells, in the uppermost chambers, are exposed to: 1) A range of attractant concentrations but no gradient (equal concentrations of peptide in the upper and

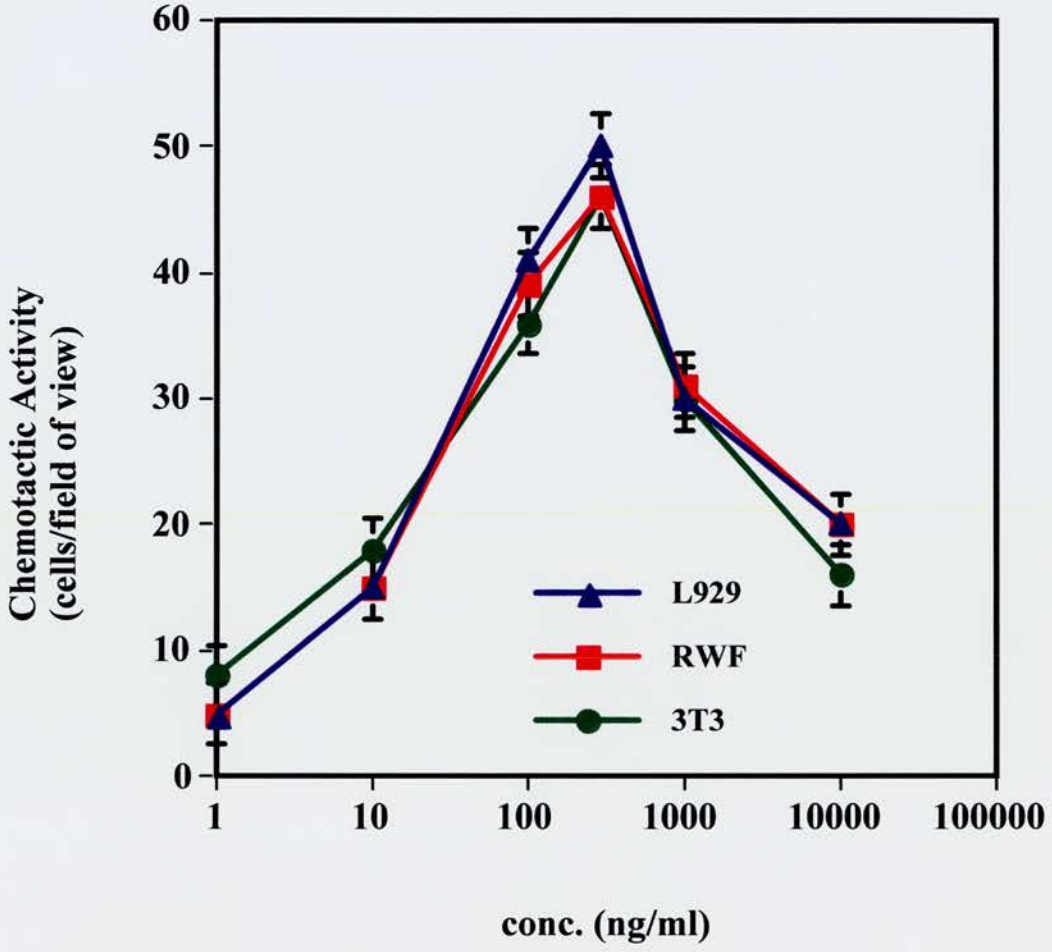

FIGURE 5.3: Chemotactic effects of the synthetic collagen peptide on different fibroblast cell lines.

The cell lines examined were L929, RWF and 3T3 fibroblasts. The synthetic collagen peptide was added to the lower compartments of a Boyden Chamber at the concentrations shown above. The net cell migration from the upper compartment was measured by counting the number of cells per field of view. Points represent the observed chemotactic activity +/- SEM for triplicate wells minus the activity of buffer control (56+/- 1.6).

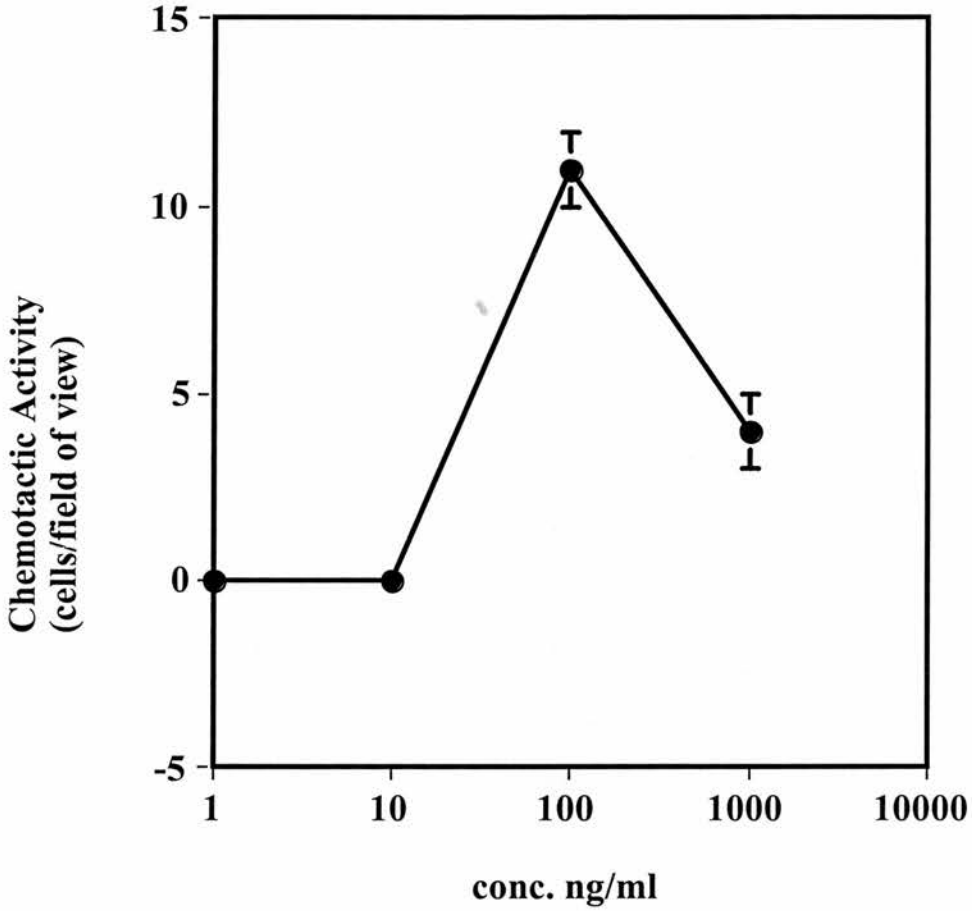

FIGURE 5.4: Effects of the synthetic collagen peptide on endothelial cell chemotaxis

The endothelial cell line examined was BAEC. The synthetic collagen peptide was added to lower compartments of a Boyden Chamber at the concentrations shown above. The net cell migration from the upper compartment was measured by counting the number of cells per field of view. Points represent the observed chemotactic activity +/- SEM for triplicate wells minus the activity of buffer control (57+/- 1.0).

lower chambers. This provides a dose-response curve for non-chemotactic effects of the peptide). 2) A range of positive gradients where the peptide concentration is always higher in the lower chamber. 3) A range of negative gradients where the peptide concentration is always lower in the lower chamber.

Chemicals that stimulate migration when placed at equal concentrations below and above the filter are characterised as affecting random motility. Attractants that are much more potent when placed below the filter are judged to elicit true directed motility.

In the top wells of the chambers, varying concentrations (0, 100ng/ml, 1μg/ml, 10μg/ml) of the collagen peptide were added to fibroblasts at a concentration of 4.8 x 10^5/ml. The same concentrations of collagen peptide were placed in the bottom wells; such that several possible concentrations above and below the filter were tested (Figure 5.5). Each combination was tested in triplicate. Filters were stained and cells counted as for the chemotaxis assay.

From the results in Figure 5.5 it can be seen that in the absence of a gradient (i.e. equal concentrations of collagen peptide in both chambers; shown in figure in bold), cell migration was still observed. This indicates that the collagen peptide is affecting random motility of fibroblast cells (chemokinesis).

In the presence of a positive gradient (i.e. higher peptide concentration in lower chamber), the number of cells migrating towards the peptide increased. In contrast, in the presence of a negative gradient (i.e. lower peptide concentration in lower chamber), the number of cells migrating through the filter decreased. These results indicate that cell migration was also directional with cells moving towards a positive concentration of collagen peptide.

	concentration above membrane			
	0	**100ng/ml**	**1μg/ml**	**10μg/ml**
concentration below membrane				
0	**58** (1.5)	60 (1.2)	60 (1.3)	63 (1.4)
100ng/ml	77 (1.1)	**63** (1.3)	62 (1.3)	65 (1.5)
1μg/ml	96 (1.1)	68 (1.2)	**66** (1.2)	68 (1.4)
10μg/ml	65 (1.6)	61 (1.3)	70 (1.4)	**70** (1.3)

Figure 5.5: "Checkerboard" analysis of cell migration to the 22-amino acid collagen peptide.
The fibroblast cell line examined was L929. In the top compartment of a Boyden Chamber, varying concentrations of collagen peptide (as above) were added together with cells. In the lower compartment the same concentrations of peptide were placed. Therefore, cells could be exposed to a range of positive and negative gradients as well as to no gradient. The net cell migration was measured by counting the number of cells per field of view. Points represent the observed chemotactic activity and standard errors (bracketed). The activity of buffer control was 56+/- 1.0.

5.6 EFFECTS ON CELL PROLIFERATION

Cell proliferation is essential to tissue repair (Chen & Abatangelo, 1999). The peptide was tested for its ability to stimulate fibroblast cell growth *in vitro.* Cells were set up in 96-well microplates and the numbers of cells present after 1, 2 and 5 days of cell growth were quantified using the Methylene Blue dye-binding assay (Section 2.5.7; Oliver *et al.,* 1989). It was found that the peptide increased growth in all fibroblast cell types (L929, 3T3, RWF; Figure 5.6). L929 growth was increased by 25% after five days, a highly significant level (high statistical significance $p<0.01$). RWF and 3T3 cell growth were increased by 12% and 15% respectively (both statistically significant, $p<0.05$).

5.7 *IN VIVO* EFFECTS

The PVA sponge animal model was used to analyse the effects of collagen peptides *in vivo* in terms of their ability to stimulate cellular infiltration and granulation tissue formation (Chapter 2.7).

PVA sponges were subcutaneously implanted into male Wistar rats and the synthetic collagen peptide was injected (100µl) into the sponges 3 days post implantation. The sponges were removed 7 and 10 days post implantation and analysed both histologically and biochemically (protein, DNA and collagen content). The peptide was tested in this assay at 4 different concentrations: 100ng/ml, 1µg/ml, 10µg/ml and 3mg/ml.

The protein content of PVA sponges containing collagen peptide was compared to control sponges (PBS buffer only) over 7 and 10 day periods, post-implantation (Figure 5.7). The level of protein recovered from sponges containing the synthetic collagen peptide (at a concentration of 10µg/ml) after both 7 and

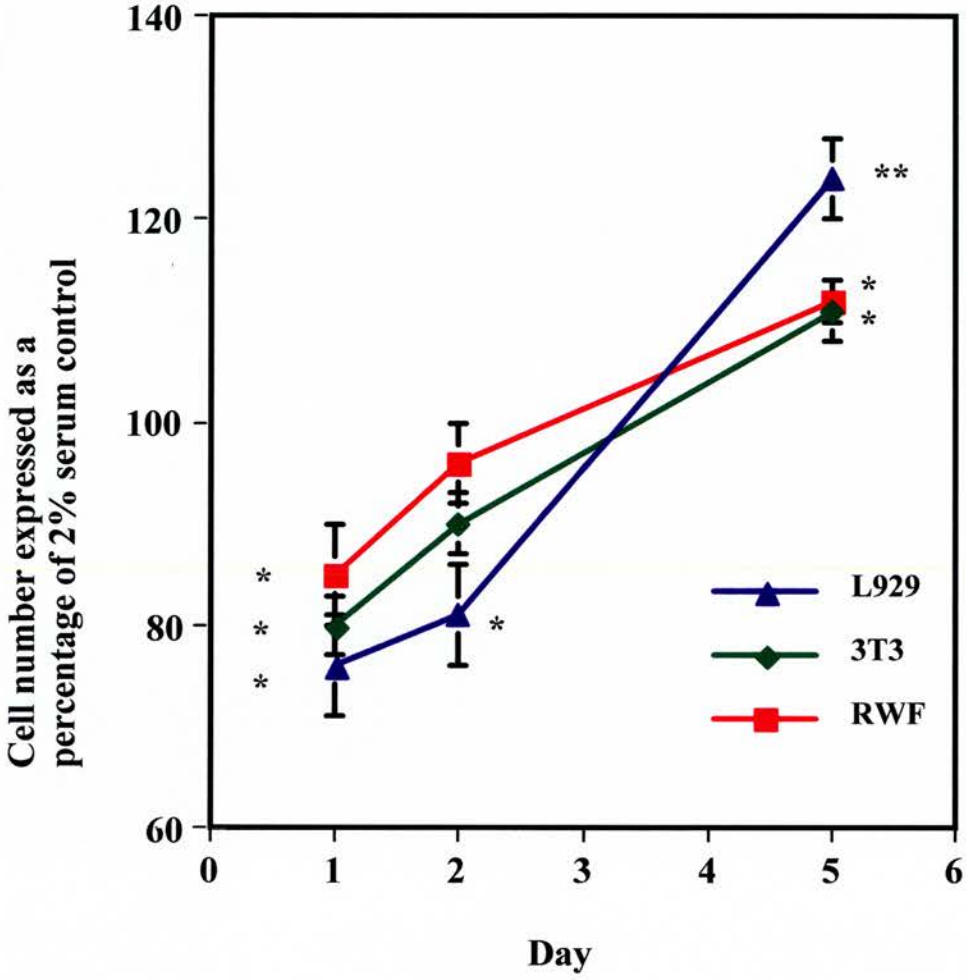

FIGURE 5.6: Effects of the synthetic collagen peptide on fibroblast proliferation

The fibroblast cell lines used were L929, 3T3 and RWF. A synthetic collagen peptide was added at a concentration of 500ng/ml and the growth in all three cell lines was measured over 5 days as shown. Cells were seeded at a density of 5000 cells/well in 96-microwell plates containing 100µl of DMEM+2% FCS. Each value represents the mean of 6 wells (Student's t-Test). Standard errors are shown, *$p<0.05$, **$p<0.01$.

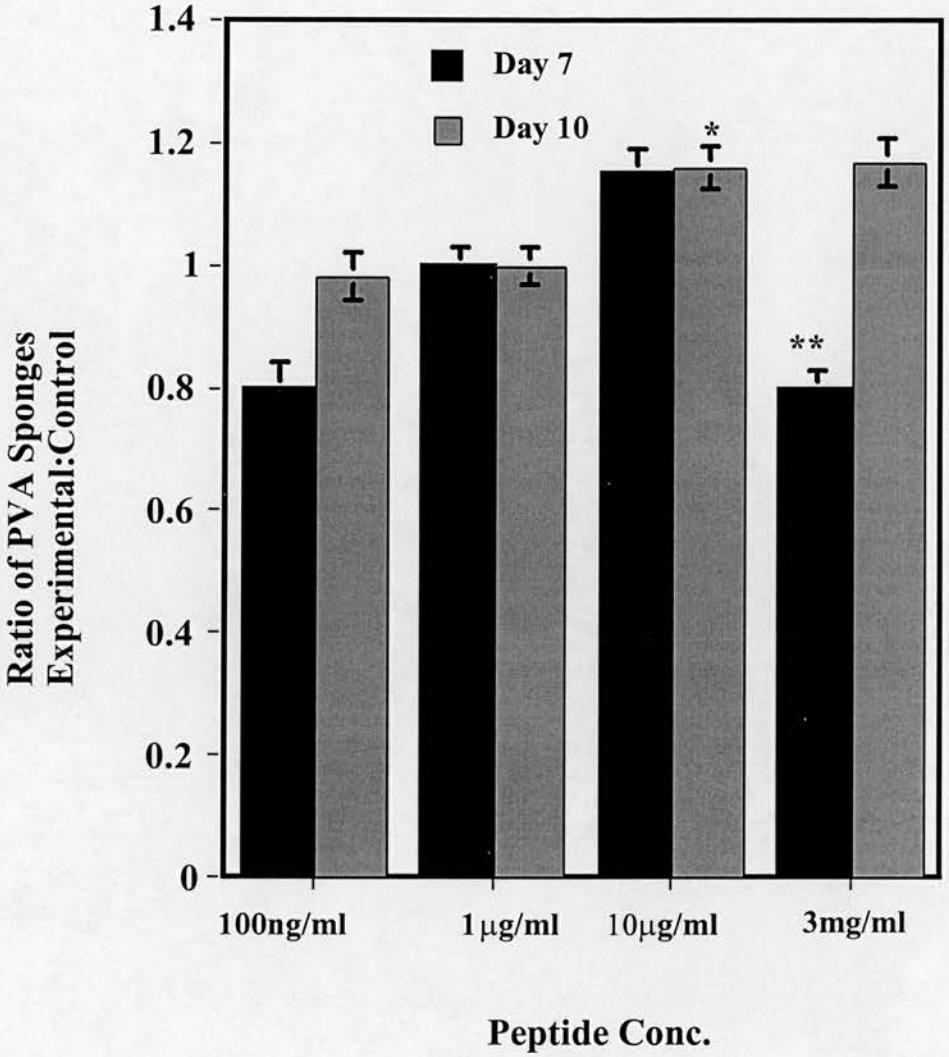

FIGURE 5.7: PVA sponge protein content

PVA sponges injected with the synthetic collagen peptide (100μl at the concentrations shown above) were removed from rats 7 and 10 days post implantation. The protein content of the sponges was determined using the Bradford Protein Assay (1976). Results are expressed as a ratio of experimental sponge (sponge + collagen peptide) to control sponge (PBS only – no collagen peptide). Standard errors are shown. *$p<0.05$, **$p<0.01$, $n=10$.

10 days was found to be 19% greater than in the control sponge. At peptide concentrations of 100ng/ml and 3mg/ml, assayed after 7 days, the protein levels within the control sponges were greater than in sponges containing collagen.

The synthetic collagen peptide had a similar, modest stimulatory effect upon cellular infiltration as indicated by the increase in DNA content of the sponges over the 7 and 10 day periods of analysis. The results (Figure 5.8) show that the amount of DNA recovered from the sponges increased in proportion to the concentration of peptide being tested, except at the highest peptide concentration (3mg/ml) where the level of DNA recovered was actually less than in the control. For the three lower peptide concentrations tested, there was a consistent increase in the amount of DNA recovered after 10 days compared to that recovered after 7 days. Under the most stimulatory conditions (10μg/ml test peptide, assayed after 10 days) the DNA content of the sponges was 20% greater than that in the control, a difference which was found to be statistically significant.

The PVA sponges injected with the synthetic collagen peptide were also analysed for collagen content, compared to control sponges. The results (Figure 5.9) show that the amount of collagen recovered from the sponges containing the peptide increased in proportion to the concentration of peptide being tested, except at the highest peptide concentration, (3mg/ml), where the level of collagen recovered was less than in controls. The peptide was most stimulatory at 10μg/ml (assayed after 10 days). The collagen content of the sponges was 21% greater than in the control, a highly significant difference. There was a consistent increase in the amount of collagen recovered after 10 days compared to that recovered after 7 days.

Each PVA sponge was also assessed for granulation tissue content by examining haematoxylin and eosin (H&E) stained sections. Granulation tissue is composed of fibrin, fibroblasts, inflammatory cells (macrophages/monocytes), matrix and blood vessels. The peptide at a concentration of 10μg/ml was shown to be stimulatory to

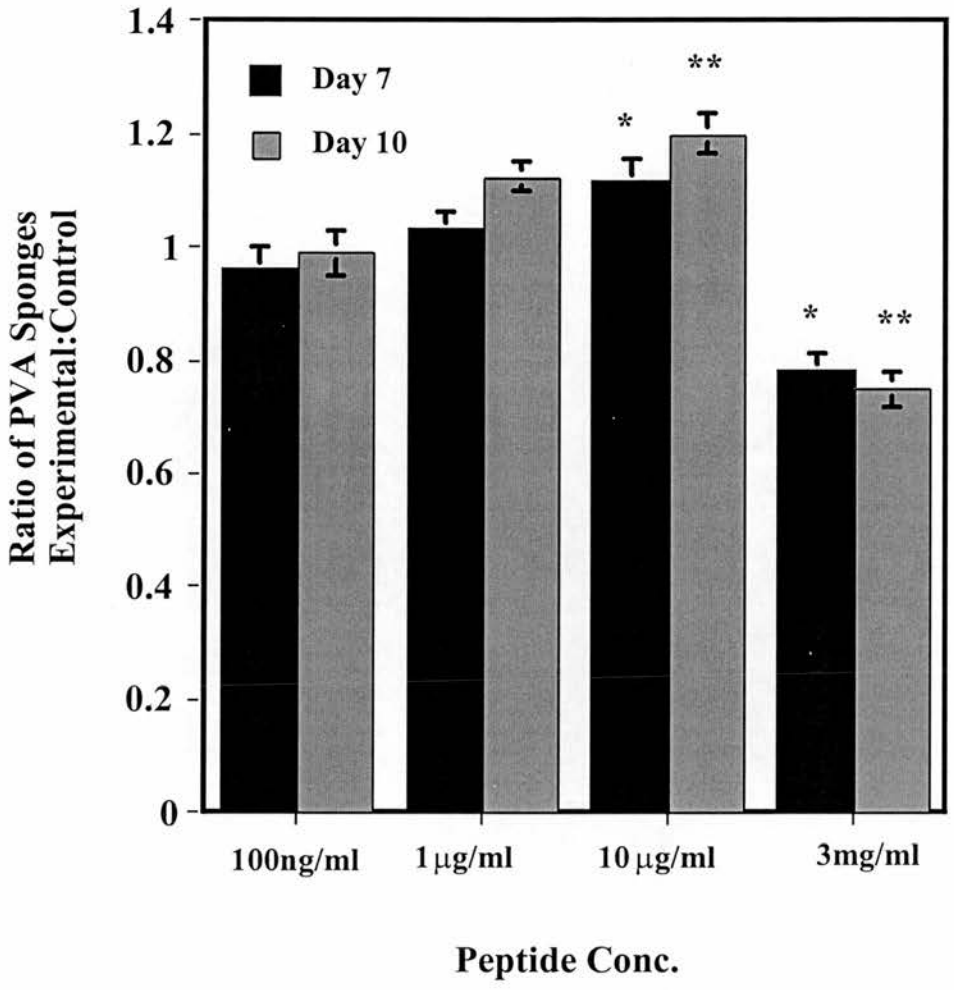

FIGURE 5.8: PVA sponge DNA content

PVA sponges injected with the synthetic collagen peptide (100μl at the concentrations shown above) were removed from rats 7 and 10 days post implantation. The DNA content of the sponges was determined using the method as described by Gendimenco *et al.,* 1988. Results are expressed as a ratio of experimental sponge (sponge + collagen peptide) to control sponge (PBS only – no collagen peptide). Standard errors are shown. *$p<0.05$, **$p<0.01$, $n=10$.

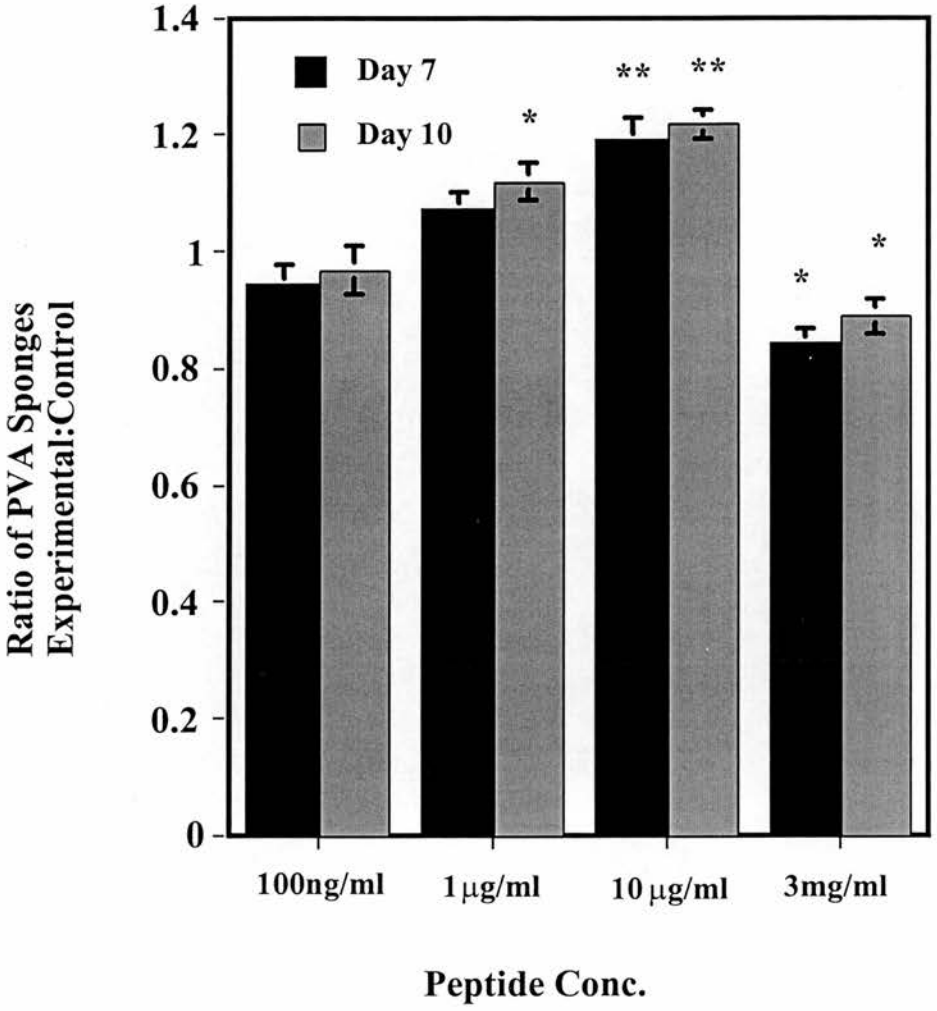

FIGURE 5.9: PVA sponge collagen content

PVA sponges injected with the synthetic collagen peptide (100μl at the concentrations shown above) were removed from rats 7 and 10 days post implantation. The protein content of the sponges was determined using the method based on hydroxyproline content and determined by HPLC (Negro *et al.*, 1987). Results are expressed as a ratio of experimental sponge (sponge + collagen peptide) to control sponge (PBS only – no collagen peptide). Standard errors are shown. *$p<0.05$, **$p<0.01$, $n=10$.

the formation of granulation tissue compared to control sponges (i.e. not injected with collagen peptide) as shown in Figure 5.10. Greater amounts of fibrin, fibroblasts and inflammatory cells have been attracted into the sponge. At a higher concentration of 3mg/ml the peptide was inhibitory to the formation of granulation tissue within the PVA sponge compared with control (Figure 5.11).

In vivo, the peptide showed effects on the accumulation of collagen, DNA and protein in PVA sponges implanted into rats and removed after 7 and 10 days post injection. Protein, DNA and collagen content of the sponges increased significantly compared with control values after 10 days. This effect was concentration-dependent, with the optimum tested concentration being 10μg/ml. At 3mg/ml the DNA, protein and collagen levels were actually lower than controls. Histological analysis of the sponges supported the findings that the collagen peptide increases the amount of granulation tissue in the PVA sponges in a dose-dependent manner. More fibrin, fibroblasts and inflammatory cells migrated into sponges injected with collagen peptide (10μg/ml) than control sponges. However, in agreement with the biochemical results, sponges containing 3mg/ml of collagen peptide inhibited formation of granulation tissue compared to control sponges.

5.8 LOCALISATION OF THE SYNTHETIC PEPTIDE'S CHEMOTACTIC DOMAIN

In order to define the amino acid sequence responsible for the chemotactic activity of the synthetic collagen peptide, a group of overlapping peptides from within the peptide was synthesised. Five peptides, each of eight amino acids in length and overlapping by 4 amino acids, were prepared and tested for their chemotactic ability *in vitro* (Figure 5.12). Peptide 4 contained the most chemotactic activity (Figure 5.13). Peptides 1, 3 and 5 were also chemotactic, but to a lesser degree (Figure 5.13). Peptide 2 was found to be least chemotactic.

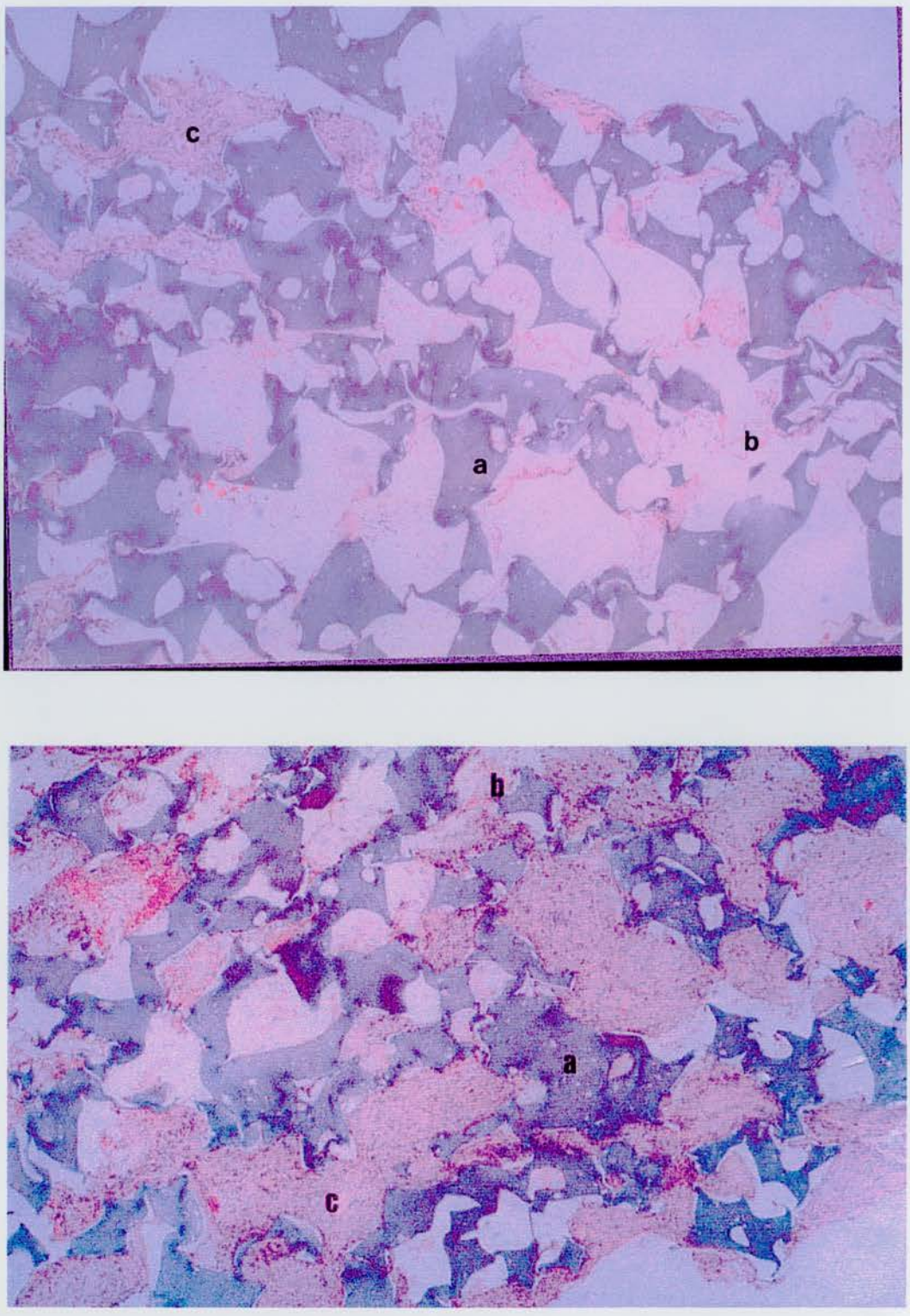

Figure 5.10: PVA sponge histology

PVA sponges were assessed for granulation tissue content in Haematoxylin and Eosin stained sections (magnification x40).

(A) control sponge (PBS only) – little cellular invasion

(B) sponge containing Collagen I peptides (10μg/ml, removed after 10 days post-implantation) – good cellular invasion.

a=PVA sponge b=fibrin c=granulation tissue

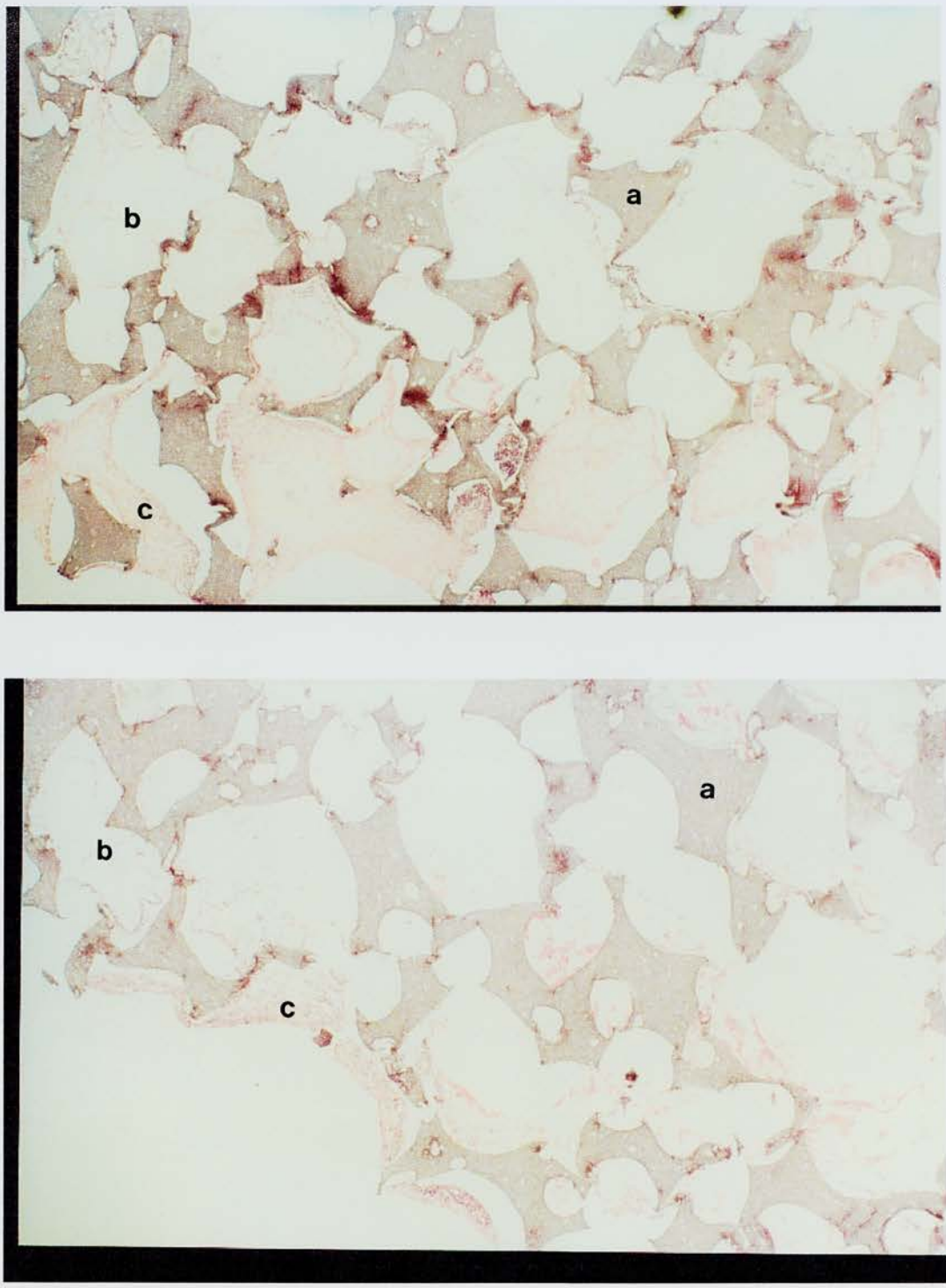

Figure 5.11: PVA sponge histology
PVA sponges were assessed for granulation tissue content in Haematoxylin and Eosin stained sections (magnification x40).
(A) control sponge (PBS only) – little cellular invasion
(B) sponge containing Collagen I peptides (3mg/ml, removed after 10 days post-implantation)– poor cellular invasion.
a=PVA sponge b=fibrin c=granulation tissue

	Residue number
Peptide 1: Gly-Pro-Hyp-Gly-Glu-Hyp-Gly-Glu	**25-32**
Peptide 2: Glu-Hyp-Gly-Glu-Hyp-Gly-Gln-Hyp	**29-36**
Peptide 3: Hyp-Gly-Gln-Hyp-Gly-Pro-Ala-Gly	**33-40**
Peptide 4: Gly-Pro-Ala-Gly-Ala-Arg-Gly-Pro	**37-44**
Peptide 5: Ala-Gly-Ala-Arg-Gly-Pro-Ala-Gly	**39-46**

Figure 5.12 : Synthetic Overlapping Peptides. 5, 8 amino acid peptides derived from the isolated 22 amino acid collagen peptide were synthesised.

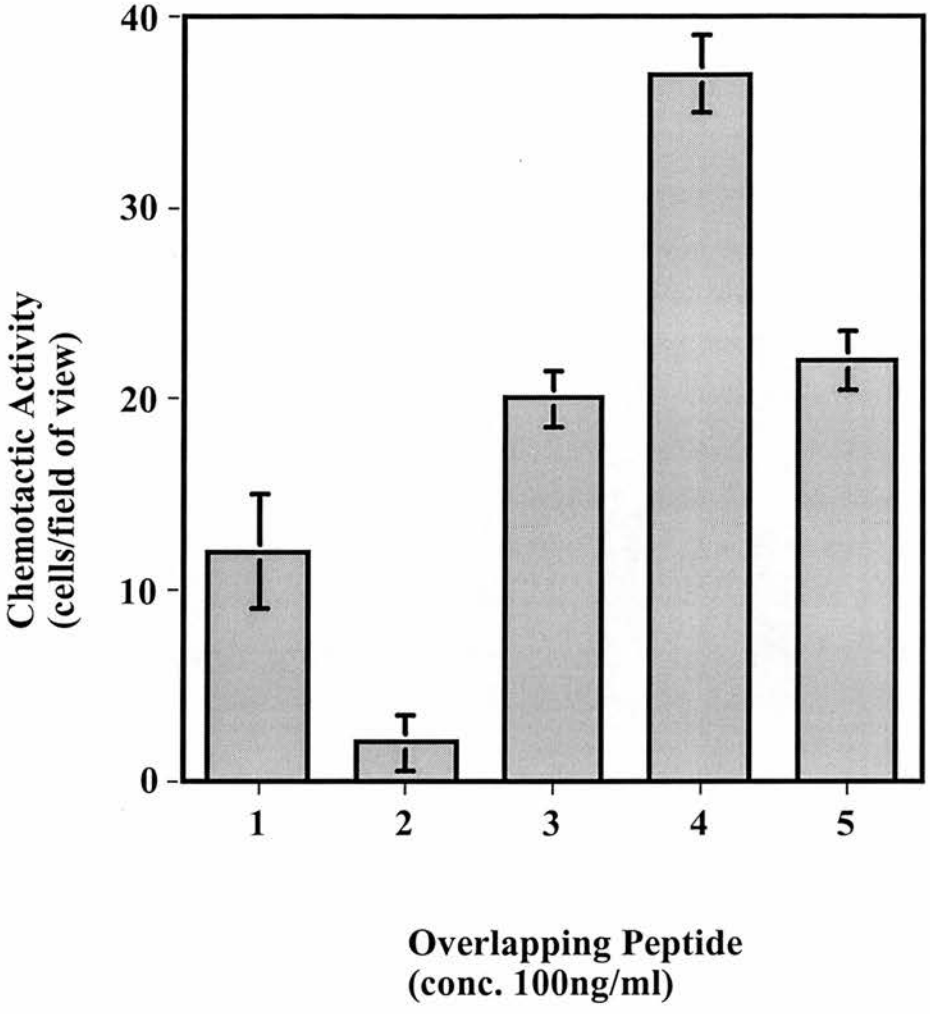

FIGURE 5.13: Chemotactic effects of 8 amino acid long overlapping peptides from 22 amino acid collagen sequence (see Fig 5.12)

Five overlapping peptides, each of 8 amino acids in length, derived from a 22 amino acid collagen peptide, were synthesised. The peptides at a concentration of 100ng/ml were added to lower compartments of a Boyden Chamber. The net cell migration from the upper compartment was measured by counting the number of cells per field of view. Points represent the observed chemotactic activity +/- SEM for triplicate wells minus the activity of buffer control (56+/-1.1).

Based on the above results, nine additional peptides, varying between 2 and 4 amino acids in length (Table 5.1), were synthesised and tested for their chemotactic ability in the Boyden Chamber Assay. Only one of these peptides, Gly-Pro-Ala-Gly, was found to contain any significant activity, stimulating chemotaxis by 64%. None of the starter peptides which comprise sub-domains of the G-P-A-G peptide (Gly-Pro, Pro-Ala, or Ala-Gly) exhibited chemotactic activity to a substantial level. These results suggest that the peptide Gly-Pro-Ala-Gly is a potent stimulator of fibroblast chemotaxis.

In order to confirm that Gly-Pro-Ala-Gly was the most chemotactic domain, smaller peptides of between 2-4 amino acids in length were synthesised and tested (Table 5.1). Gly-Pro-Ala-Gly was the only peptide stimulating chemotaxis to any significant level. From these results, the importance of the sequences surrounding a chemotactic domain can be seen, as these may be inhibit chemotaxis.

5.9 INVESTIGATION OF POSSIBLE CELL SURFACE RECEPTORS

As previously mentioned (see Chapter 1.6), cell surface receptors of the integrin family are known to bind regions of the collagen molecule. Integrin types $\alpha_1\beta_1$, $\alpha_2\beta_1$ bind to collagen (Knight *et al.*, 2000),. although the actual form of the collagen molecule is crucial to binding (Tuckwell *et al.*, 1996). Triple helical collagen is known to interact in the binding to these receptors, but it is still unclear whether or not non-triple helical collagen peptides will bind to the receptors. It is thought that collagen peptides use another form of receptor binding which is not integrin mediated (Tuckwell *et al.*, 1996).

In order to investigate whether the binding of collagen was integrin mediated, antibodies to the integrins were employed to block the binding process in the Boyden Chamber Chemotaxis Assay. The antibodies used were integrin specific mouse monoclonals. If the process were integrin-dependent then the chemotactic

Peptide tested	Chemotactic activity (mean no. cells/field of view +/- SEM) n=9
Gly-Pro-Hyp-Gly	3+/-1.2
Gly-Pro-Ala-Gly	44+/-2
Gly-Pro-Glu-Gly	2+/-1.4
Gly-Pro-Hyp	6+/-1.4
Gly-Pro-Ala	9+/-1.3
Pro-Ala-Gly	4+/-1.8
Gly-Pro	3+/-1.1
Pro-Ala	5+/-1.0
Ala-Gly	2+/-1.2

Table 5.1 : Chemotactic effect of synthesised collagen peptides on L929 fibroblasts.

Collagen peptides 2-4 amino acid long (conc. 50ng/ml) were examined for chemotactic potency. Points represent the observed chemotactic activity +/- SEM for triplicate wells minus the activity of buffer control (56.6+/- 1.3).

activity of collagen peptides should be impaired in this assay. Pepsin solubilised collagen (PSC) was used as a triple-helical collagen control and the synthetic 22-amino acid collagen peptide as a non-triple helical test. From the results in Figure 5.14 it can be seen that the antibodies to integrins $\alpha_1\beta_1$ and $\alpha_1\beta_2$ inhibited chemotaxis by the triple helical collagen whilst antibodies to $\alpha_1\beta_3$ integrin had no effect. Under these conditions the antibodies had no-effect upon the activity of the synthetic collagen peptide.

Since the collagen peptide was at a higher molarity than the triple helical collagen, it is possible that the integrin antibody concentration was not high enough to inhibit the peptides. Therefore, the antibodies were tested at higher concentrations (Figures 5.15, 5.16, and 5.17). These results showed that even at the highest concentration (1:1) the integrin antibodies did not block the chemotactic effects of the collagen peptide.

5.10 SUMMARY

The biological activities of a chemically synthesised 22-amino acid collagen peptide, previously isolated (Chapter 4), were examined. This synthetic collagen peptide was found to stimulate chemotaxis in three fibroblast cells lines, but was not significantly chemotactic for endothelial cells. The chemotactic domain of this peptide was localised. The tetra peptide Gly-Pro-Ala-Gly was found to be the minimum sequence essential in order to elicit a chemotactic response.

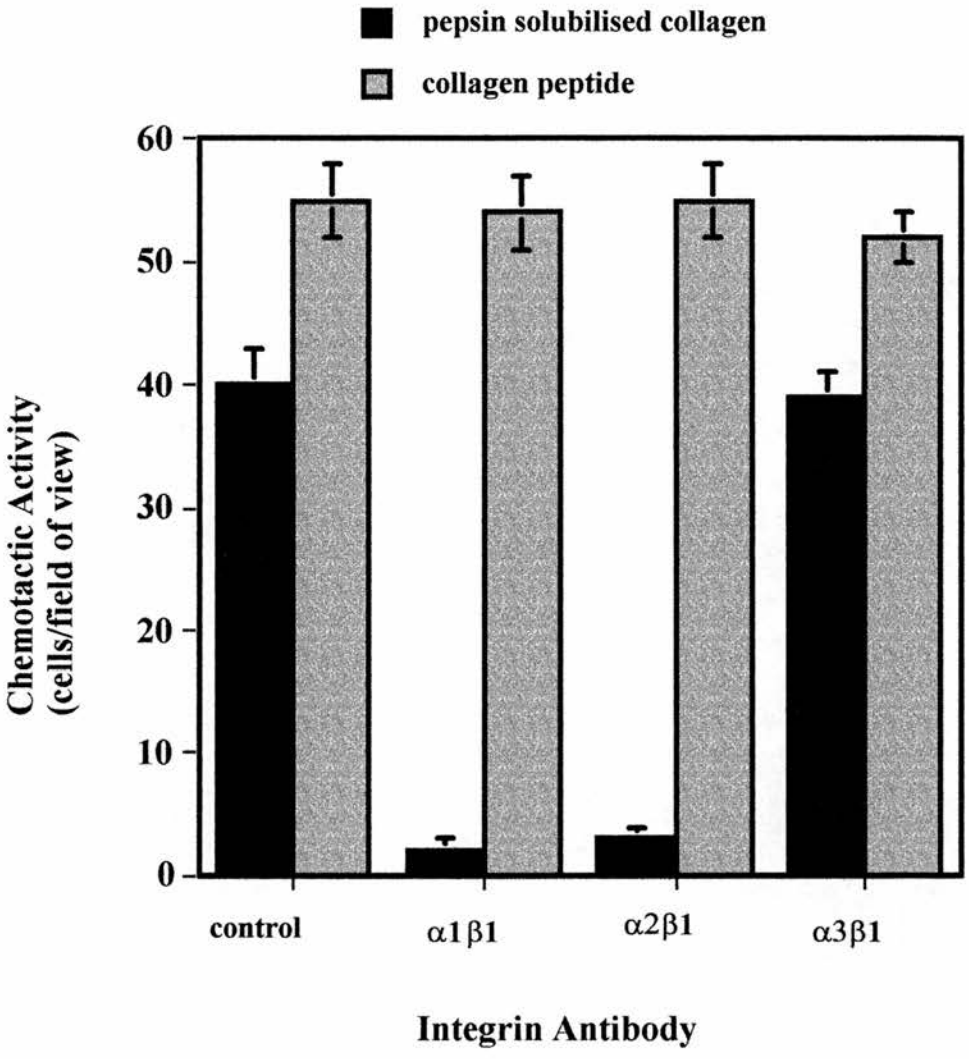

FIGURE 5.14: Effects of integrin antibodies on collagen chemotaxis induced by triple-helical and non-triple helical collagen.
Triple helical collagen (pepsin solubilised) and non-triple helical 22 amino acid collagen peptide were examined for their effects on L929 fibroblast chemotaxis, with the addition of integrin antibodies. The collagen/peptides at a concentration of 500ng/ml and the antibodies at a dilution of 1:500 were added to lower compartments of a Boyden Chamber at the concentrations shown above. The net cell migration from the upper compartment was measured by counting the number of cells per field of view. Points represent the observed chemotactic activity +/- SEM for triplicate wells minus the activity of buffer control (56+/- 1.8).

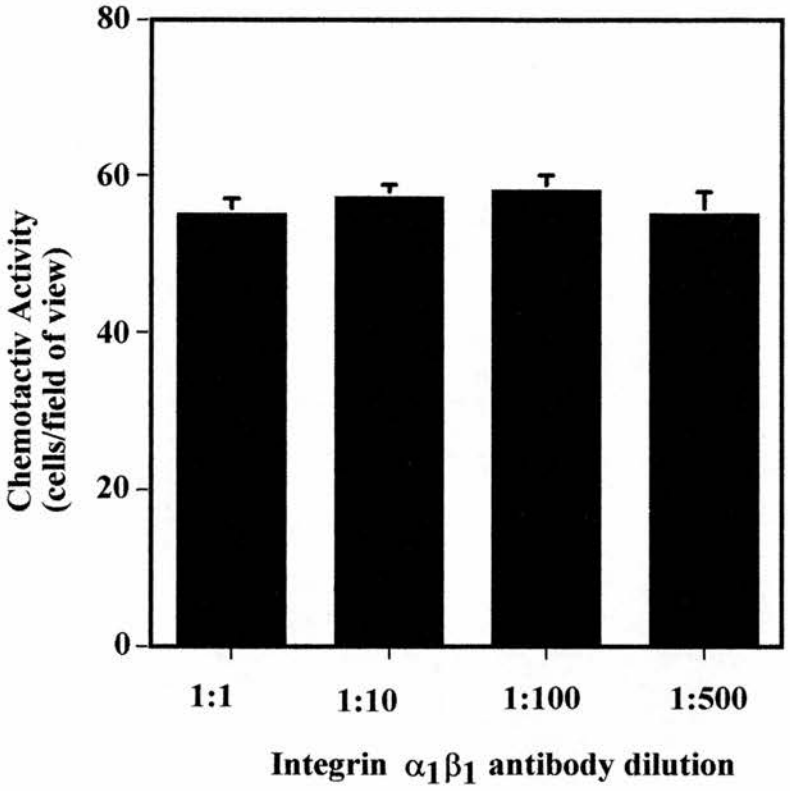

FIGURE 5.15: Effects of $\alpha_1\beta_1$ antibody concentration on collagen peptide-mediated chemotaxis.

The antibody to the integrin $\alpha_1\beta_1$ was examined for its ability to block L929 fibroblast chemotaxis. Collagen peptide conc. 500ng/ml. The antibody at the dilutions shown above was added to lower compartments of a Boyden Chamber at the concentrations shown above. The net cell migration from the upper compartment was measured by counting the number of cells per field of view. Points represent the observed chemotactic activity +/- SEM for triplicate wells minus the activity of buffer control (56+/- 1.5).

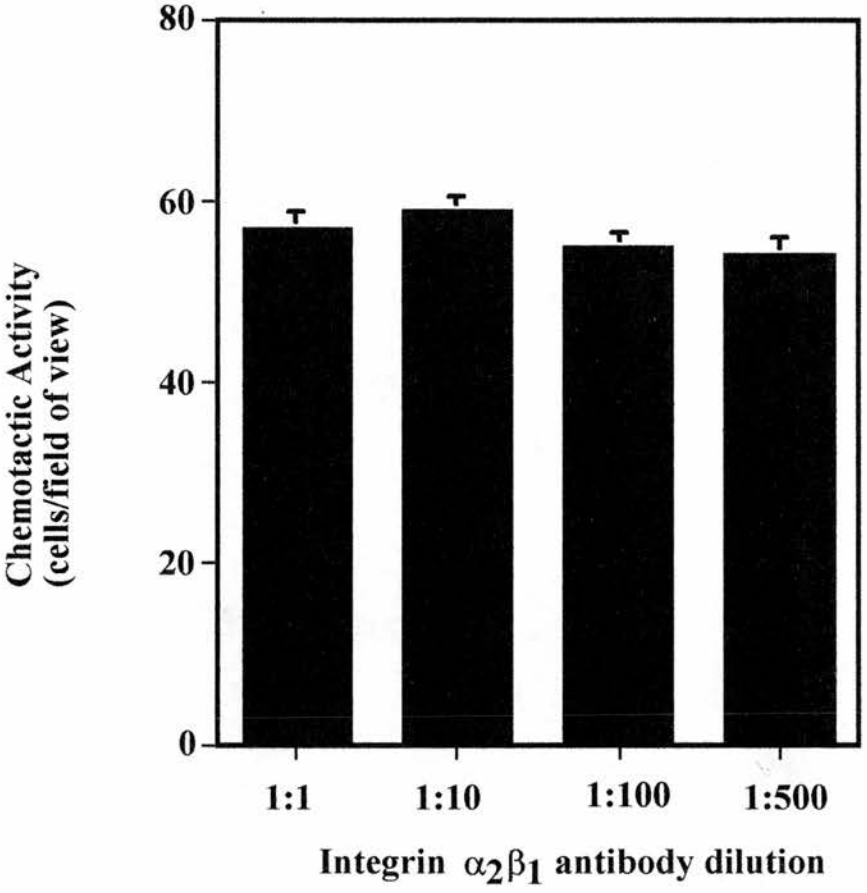

FIGURE 5.16: Effects of $\alpha_2\beta_1$ antibody concentration on collagen peptide-mediated chemotaxis.

The antibody to the integrin $\alpha_2\beta_1$ was examined for its ability to block L929 fibroblast chemotaxis. Collagen peptide conc. 500ng/ml. The antibody at the dilutions shown above was added to lower compartments of a Boyden Chamber at the concentrations shown above. The net cell migration from the upper compartment was measured by counting the number of cells per field of view. Points represent the observed chemotactic activity +/- SEM for triplicate wells minus the activity of buffer control (56+/- 1.5).

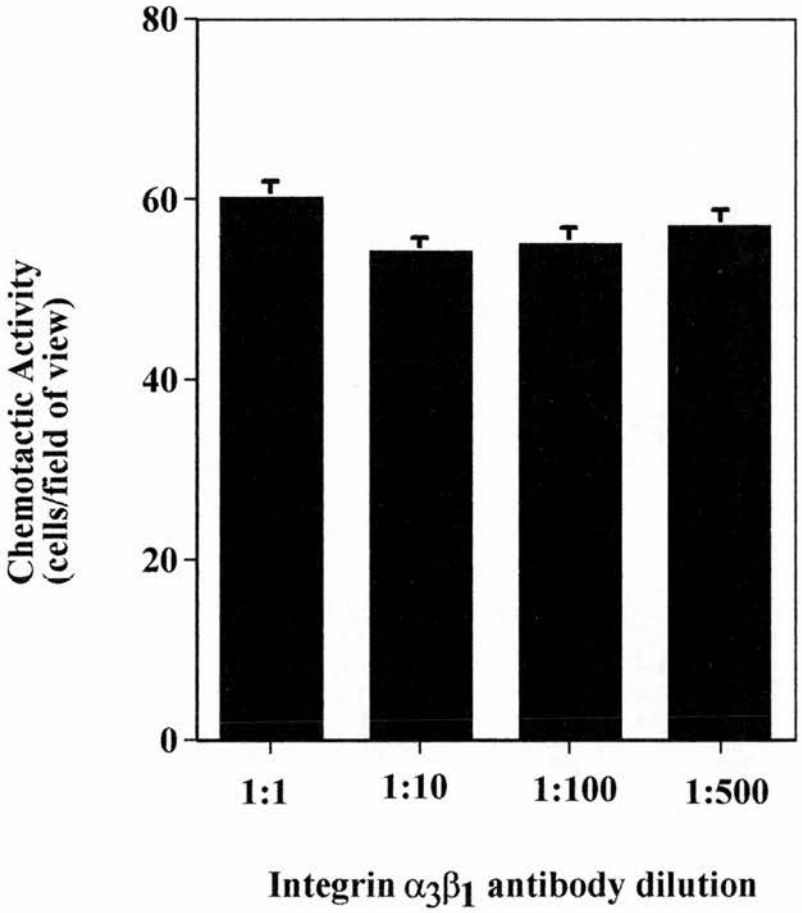

FIGURE 5.17: Effects of $\alpha_3\beta_1$ antibody concentration on collagen peptide-mediated chemotaxis.

The antibody to the integrin $\alpha_3\beta_1$ was examined for its ability to block L929 fibroblast chemotaxis. Collagen peptide conc. 500ng/ml. The antibody at the dilutions shown above was added to lower compartments of a Boyden Chamber at the concentrations shown above. The net cell migration from the upper compartment was measured by counting the number of cells per field of view. Points represent the observed chemotactic activity +/- SEM for triplicate wells minus the activity of buffer control (56+/- 1.8).

CHAPTER 6:

DISCUSSION

6.1 BACKGROUND

Wound healing is a complex response to tissue injury which is essential to the survival of living organisms. The repair process encompasses several events with different cell types, cytokines, coagulation factors and matrix proteins involved (reviewed by Clark 1993). The extracellular matrix (ECM) was initially thought only to function as a support, however many of the matrix components have now been identified as having a variety of additional biological properties (Raghow 1994). The major component of the ECM is collagen, a glycoprotein composed of three polypeptide chains based on the general structure Gly-X-Y (see Section 1.3.1; van der Rest & Garrone 1991). Collagen has been employed in the formulation of medical materials (Pachence 1996) due to its good safety profile and its ready and cheap availability. It has uses as a dressing for burns, pressure sores and leg ulcers (Chvapil *et al.*, 1986). Collagen membranes have also been used as drug carriers (Miyata *et al.*, 1979), and in an injectable form, zyderm collagen implant (ZCI) has been used as an implant for replenishing natural skin collagen (Ruszczak & Schwartz 1999). These commercially successful collagen-based medical devices all use collagen's physical rather than biological properties. These biological properties of collagen have been shown to be involved in haemostasis (Morton *et al.*, 1987), cell attachment (Anderson 1992), cell proliferation (Thoumie *et al.*, 1995) and cell migration (Postlethwaite & Kang 1976). During the wounding process, the extracellular matrix is degraded, forming breakdown products, and these breakdown products have been shown to play a role in wound healing. Collagen and collagen-derived peptides have chemotactic properties, stimulating the migration of neutrophils, monocytes, macrophages and fibroblasts (Postlethwaite *et al.*, 1978; Malone *et al.*, 1991). Cyanogen bromide (CNBr)-derived collagen peptides effect the attachment and migration of neural crest cells (Perris *et al.*, 1993).

It was the aim of this study to further investigate collagen's biological role in the context of wound healing. It was decided to isolate collagen peptides with the most

biologically active properties. These peptides could then be assessed for their abilities to enhance the healing process *in vivo.*

6.2 BIOLOGICAL EFFECTS OF COLLAGEN AND COLLAGEN-DERIVED PEPTIDES

Collagen comprises nineteen different types (I-XIX; Sakai 1995). More than 90% of all fibrous protein is Type I Collagen (Pachence 1996) and it is the most abundant collagen type in skin (Trojanowska *et al.*, 1997). Collagens III and V are the other predominant collagen types in skin. In this study Collagens I, III and V were purified by a variety of methods and their peptides generated. Collagens I and III were isolated from pig skin, a cheap and readily available source. Type V was purified from pig placenta. Other collagen types could have been isolated, however finding a convenient source would have been outwith the scope of this study (see Table 1.1). In the first part of this study, predominantly Types I and III were studied. Later, all three were investigated.

6.2.1 Collagens I and III – CNBr-derived peptides

The biological effects of CNBr peptides derived from collagen were examined in Chapter 3. Collagen peptides can be generated by many methods [CNBr, matrix metalloproteinases (MMPs), various collagen degrading enzymes]. MMPs are very good natural proteases, however they are not readily available commercially and their isolation is difficult, hence they were not used here. In this investigation CNBr digestion was used as collagen peptides derived from CNBr digestion had already been shown to have biological activities (Perris *et al.*, 1993; Saelman *et al.*, 1993). CNBr and enzymatic digestion (see Section 2.3) are very cheap and rapid ways of generating peptides.

Collagens I and III were purified from pig skin and the major CNBr peptides [α1(I) CB3, 6, 7, 8 and α1(III) CB3, 4, 5, 8] isolated by FPLC (Sections 3.2-3.4). In Collagen I these major peptides were isolated from the α1 chain only. The effects of these peptides on fibroblast and endothelial cell proliferation, and chemotaxis were investigated. Three different cell lines were examined; L929 fibroblasts, rat wound fibroblasts (RWF) and bovine aorta endothelial cells (BAEC). L929 cells, derived from an established cell line, are robust and easy to handle and were used in the setting up and standardisation of the assays used. RWF are primary cell lines and hence were used as they are nearer to the natural environment found at the wound site. BAEC are a primary endothelial cell line. Both fibroblasts and endothelial cell types are essential to wound healing. Cell proliferation was assessed by using the Methylene Blue Dye-Binding Assay (Oliver *et al.*, 1986). Chemotaxis was measured in the Boyden Chamber Assay (Falk *et al.*, 1980).

The CNBr-derived peptides were isolated by cation-exchange FPLC (Figures 3.5 & 3.6) as for the method of Bateman *et al.*, 1986. Peptides were analysed by SDS-PAGE to confirm identity and purity (Figures 3.3 & 3.4). This method resolves all the major peptides in a rapid and reproducible fashion. However, purity levels did vary with each peptide. Collagen I peptides CB7 and CB8 contained some incompletely cleaved fragments. In retrospect, these partially cleaved peptides could have been further separated by gel permeation chromatography. The purity of Collagen III peptide CB5 could also have been improved if the FPLC gradient was run over a longer period of time (i.e 120 mins). This would have helped to separate the peaks on the FPLC trace (Figure 3.6).

The Methylene Blue Dye-Binding Assay (Oliver *et al.*, 1989) was used to quantify cell numbers after growth in the presence of collagen-derived peptides. This is a reproducible, rapid and inexpensive method. However, it does have limitations. The automated microplate reader for measuring optical densities of cells restricts the range of cell numbers that can be quantitated. Preliminary experiments to optimise the

assays were carried out in order to minimise this effect. Hence, cells were grown in DMEM containing 2% serum. Cells grown in 10% serum reached levels which could not be measured accurately using this method.

Cell migration was measured using the Boyden Chamber Assay (Falk *et al.*, 1989). It is a simple and rapid assay. Cells responding to a concentration gradient of collagen peptide diffuse towards the peptide through a membrane filter. Cells reaching the bottom surface of the membrane are fixed, stained and counted. A limitation of this method is that some cells may not adhere to the membrane and will 'fall off'. Reducing incubation time to 2.5 hours minimised this effect.

6.2.1.1 Effects of CNBr-derived peptides on cell proliferation

It was found that Collagen I peptides CB3, CB8 & CB7 and Collagen III peptides CB4 & CB8 stimulated cell proliferation. These results are summarised in Table 6.1, column 2.

Peptide CB8 from Collagen I, increased BAEC cell growth/proliferation by 60%, at a concentration of 10μg/ml after 5 days. CB3 from Collagen I, at a concentration of 100μg/ml increased growth of both BAEC and RWF cells by 20% after 5 days. Peptide CB7 at 100μg/ml also increased growth of RWF cells by 20%. CB6 had no significant effect on cell proliferation. CB4 from Collagen III increased L929 growth by 35% at a concentration of 1ng/ml. CB8 from Collagen III also at a concentration of 1ng/ml increased growth by 19%. CB5 and CB3 α1(III) had no significant effects on cell proliferation.

Therefore, it was shown that although CB8 α1(I), at a concentration of 10μg/ml produced the greatest increases in growth, CB4 α1(III) was more potent being active at a lower concentration (1ng/ml). These effects were concentration-dependent, which will be discussed in Section 6.4.

Peptide	Maximum % increase in cell proliferation			Maximum % increase in cell chemotaxis		
	Cell Type	Optimal Concentration	% Cell Proliferation	Cell Type	Concentration	% Cell Chemotaxis
Collagen α1(I)						
CB3	RWF	100μg/ml	20	L929	1μg/ml	62
CB6	nd	nd	nd	L929	1mg/ml	30
CB7	RWF	100μg/ml	20	L929	1mg/ml	35
CB8	BAEC	10μg/ml	60	L929	1mg/ml	64
Collagen α1(III)						
CB3	nd	nd	nd	L929	1mg/ml	25
CB4	L929	1ng/ml	35	L929	1mg/ml	44
CB5	nd	nd	nd	L929	1mg/ml	15
CB8	BAEC	1ng/ml	19	L929	1mg/ml	45

nd – denotes on all cell types no cell proliferation was detected

Table 6.1: A summary of the effects of CNBr-derived collagen peptides on cell proliferation and chemotaxis

The maximum effects on cell proliferation and chemotaxis by Collagen I and III CNBr-derived peptides are shown. Potency levels are as indicated.

6.2.1.2 Effects of CNBr-derived peptides on cell chemotaxis

All of these isolated CNBr peptides were shown to be chemotactic to some extent. α1(I) CB3, α1(I) CB8, α1(III) CB4 and α1(III) CB8 were the most chemotactic peptides isolated. These results are summarised in Table 6.1, column 3.

α1(I) CB3 showed the greatest effect, increasing chemotaxis by 62% above control levels, at a concentration of 1ng/ml. α1(I) CB8 increased chemotaxis to a similar degree, however was found to be less potent, being active at a concentration of 1mg/ml. CB4 and CB8 α1(III) increased chemotaxis by 44% and 45% respectively, at a concentration of 1mg/ml.

As mentioned, a number of chemotactic factors have been found in wounds and it is thought that they act as attractants to regulate the number of cells and the order in which they enter the wound, with each cell type responding only to specific attractants. Chemoattractants show an enormous range in their effective doses *in vitro*, suggesting different functions *in vivo*. Collagen peptides could create a low and prolonged signal in the presence of tissue debris and bring in phagocytic cells for continuing clean up and also fibroblasts to repair damaged tissue. More potent molecules such as TGF-β would be expected to diffuse into the surrounding tissue and reach their cellular target more efficiently.

6.2.2 Collagens I, III and V – enzyme-derived peptides

Collagens I, III and V were degraded by enzymatic digestion (see Chapter 4). The enzymes used were collagenase, trypsin and chymotrypsin. The peptide mixtures generated (from α1 and α2 chains where applicable) were tested for chemotactic activity in the Boyden Chamber Assay. Many regions of the collagen chain (from different collagen types) were shown to be capable of eliciting a chemotactic response (Figure 4.1). It was demonstrated that peptides produced by bacterial collagenase of

digestion of collagens I and III were chemoattractants for fibroblasts (Tables 4.3-4.4).

However, the work described in this chapter shows that the degree of chemotactic potency depends on both the collagen type and the digestion method employed. Peptide mixtures derived from Collagen I by a double digestion with bacterial collagenase and chymotrypsin proved to be the most potent chemoattractant, increasing chemotaxis by three times that of control levels. Chemotaxis was again found to be concentration-dependent.

The optimal conditions for producing chemotactic peptides were determined in Chapter 4 (temperature, time, enzyme concentration). These were found to be a digestion time of 24hrs, at 37°C, with an enzyme concentration of 10μg/ml. It was found that Collagen I digested with collagenase and chymotrypsin produced peptides with the greatest chemotactic effects, compared to the other collagen types tested (Figure 4.1). Hence, Collagen I peptides were focused upon in subsequent experiments. The most bioactive peptide from Collagen I was isolated by HPLC. This peptide was found to be a potent chemoattractant. Its sequence was determined and was found to be 22 amino acids in length and corresponded to the α2 chain of collagen (residues 25-46). This work is summarised in a schematic representation in Figure 6.1.

The work in this study was in agreement with Postlethwaite *et al.* (1978), who showed that Collagens I and III after bacterial collagenase digestion were fibroblast chemoattractants. In contrast Albini & Adelmann-Grill (1985) demonstrated that digestion with bacterial collagenase abolished the chemotactic activity of collagen. Different experimental conditions used could be a possible explanation for these conflicting results i.e. variations in buffers, cell lines, enzymes.

In vivo, collagen is degraded by various enzymes after wound formation. Initially, mammalian collagenase (MMP 1) cleaves the collagen molecule across the three

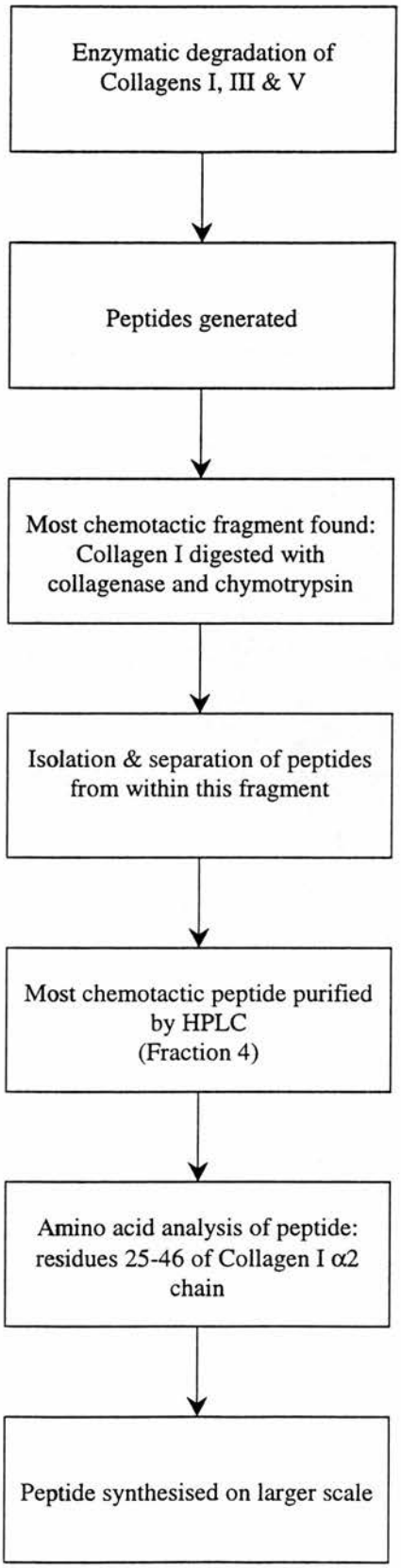

FIGURE 6.1:
Schematic representation of the isolation of a bioactive peptide from Collagen I

chains to give two triple helical fragments. These fragments become denatured at physiological temperature and the denatured chains are readily digested by most proteases (Figure 1.2). Therefore in a wound, many sizes of collagen peptide are found, some even thought to be as small as three amino acids in length (Postlethwaite & Kang 1976). These initial *in vitro* studies are relevant to what is thought to occur during collagen degradation in a wound, since a variety of collagen peptides were generated (Chapter 4).

6.3 BIOLOGICAL EFFECTS OF A SYNTHETIC COLLAGEN PEPTIDE

A 22-amino acid collagen peptide from Collagen I was isolated as discussed in Chapter 4. This peptide sequence was synthesised chemically on a large scale, using FMC/^{t}Bu based method of synthesis.

The biological activities of the peptide were examined and its chemotactic domain localised. This work is summarised in schematic form in Figure 6.2.

6.3.1 Chemotactic effects

The synthetic collagen peptide stimulated chemotaxis in the three fibroblast cell lines (L929, 3T3, RWF) to similar extents. However, the peptide was not significantly chemotactic for endothelial cells (see Section 6.4.4).

In order to verify that the cell migration, promoted by the peptide, was directional (chemotaxis), the 'Checkerboard Assay' (Zigmond & Hirsch 1973) was carried out. This method is used to distinguish between directional (chemotactic) and random (chemokinetic) migration. The cell migration stimulated by this peptide was shown to be both random and directional in nature.

In order to define where the activity resides in the amino acid sequence of this synthetic collagen peptide, a group of overlapping peptides from within the peptide were synthesised. One peptide was found to contain more chemotactic activity than the others. Based on this result, nine additional di and tri peptides were synthesised. None of these shorter peptides exhibited chemotactic activity to any substantial level. From this it was concluded that a tetra-peptide, and more specifically Gly-Pro-Ala-Gly was the minimum sequence essential to eliciting a chemotactic response. From these results the importance of sequences surrounding a chemotactic domain were shown. Some were found to be inhibitory, others stimulatory. The Gly-Pro-Ala-Gly

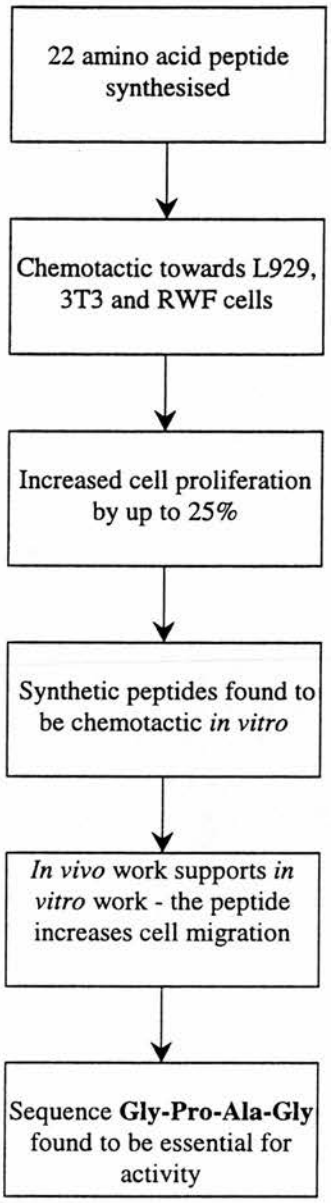

FIGURE 6.2:
Schematic representation of the synthetic collagen peptide's properties

sequence occurs several times throughout the collagen molecule. However other peptides containing this sequence were not isolated by HPLC as they were not found to be chemotactic, which supports this theory. This would indicate that an important area in chemotaxis is not only the sequence but the context.

6.3.2 Effects on cell proliferation

The peptide increased cell proliferation in all fibroblast cell types tested (L929, 3T3, RWF). Cell proliferation/growth in these cell lines increased by 25%, 15% and 12% respectively (Section 5.6, Figure 5.6), after 5 days at a concentration of 500ng/ml. This indicates that these cells types have cell-surface receptors which are recognised by collagen-peptides.

6.4 CELL-SURFACE INTERACTIONS : RECEPTORS

A major role of the ECM is in the regulation of cell/cell communication (MacNeil 1994). Specific attachment sequences on ECM proteins are recognised by cell surface receptors (Yamada & Geiger 1997).

6.4.1 Chemotaxis

The first event in chemotaxis is the detection of the chemoattractant by specific cell surface receptors (Martin *et al.*, 1983). Cells lacking such receptors are unresponsive (Wilkinson 1988). All stimulatory peptides must bind to a receptor on the host cell in order to be biologically active. The mechanisms by which collagen and its degradation products attract fibroblasts and cause migration has not been clarified. Even though different collagen peptides, different cell types and different effects on cells have been examined, the same receptor-ligand binding principle is valid. The collagen peptides have a sequence which is recognised by the cell surface receptor. Binding then occurs which sets off a variety of intracellular actions eventually resulting in changes to the cell. The results in this thesis show that different sequences within the collagen peptides are involved in the stimulation of different activities (migration, proliferation, chemotaxis etc.). There is also the possibility that different cellular receptors may be involved (see below). Although native Collagens I, III and V had some chemotactic behaviour (Tables 4.3-4.5), the peptides generated from these collagens displayed, in general, elevated chemotactic potency. In certain circumstances sequences which may be recognised by the cell may get blocked or shielded (Postlethwaite *et al.*, 1978). However, after tissue damage, when the collagen becomes degraded, sites on the molecule may be 'unshielded', so as to permit binding to cells and provide a stronger chemotactic stimulus (Malone *et al.*, 1991). It may be for this reason that the native collagen molecules were not as chemotactic as some of their derived peptides.

6.4.2 Concentration-dependence

It was found that with both chemotaxis and cell proliferation, the effects of the collagen peptides on cell behaviour were concentration-dependent. This is in agreement with work already done in this field (Postlethwaite *et al.*, 1978; Malone *et al.*, 1991). The chemotactic response is usually a concentration dependent process, producing a typical bell-shaped response curve. This effect is well known in the literature (Postlethwaite *et al.*, 1976; Albini & Adelmann-Grill 1985). The concentration effect is due to the degree of receptor occupancy (Grotendorst 1984). Cells respond to the first hit (signalled event) by the redistribution of receptors to the point of hit, this occurs by polarisation with the first hit point at the front of the cell. At low concentrations only a minority of cells polarise but they polarise well. As the concentration of chemoattractant is raised to an optimum, the proportion of responding cells rises to almost 100%. At supra-optimal concentrations the cells protrude more than one pseudopod and therefore polarise poorly. Hence, once the ligand concentration is raised above optimum, receptors become occupied simultaneously at many points on the cell surface and the cell is unable to redistribute receptors and show clear polarisation (Wilkinson & Haston 1988).

6.4.3 Chemotaxis v Proliferation

It is likely that different peptide sequences and different cell receptors are involved in chemotaxis and proliferation, since the CNBr peptides do not react in the same ways for these two cell activities. For example, CB5 α1(III) had no effect on cell proliferation (see Section 3.6.2, Tables 3.22-3.24, Figure 3.18) but increased cell chemotaxis by 30% (see Section 3.8, Table 6.1). Perhaps different sequences on the collagen molecule are needed to switch on these activities. A specific sequence on the molecule may bind to a cell receptor and stimulate the cell to move into the wound site and then another different sequence may be involved in stimulating the cell to grow.

Peptides α1(I) CB3 and α1(I) CB8 were the CNBr fragments which stimulated fibroblast cell growth (see Section 3.6.1, Tables 3.1-3.6, Figures 3.7 & 3.9) and chemotaxis (see Section 3.8, Figures 3.21-3.22), suggesting that these fragments contain binding sites recognised by the fibroblasts. These peptides did not, however, stimulate growth and chemotaxis to the same extent, which implies that different receptors and recognition sites are involved in the interactions. CB4 and CB8 were the Type III collagen peptides which stimulated fibroblast growth (see Section 3.6.2, Tables 3.13-3.18, Figures 3.13 & 3.15) and chemotaxis (see Section 3.8, Figures 3.23-3.24), again the degrees of stimulation varied with the two fragments, suggesting similar cell-collagen interactions as the Type I peptides.

An example of similar effects in the literature have been shown by Watson & Gibbins, 1998. Studies on cyanogen bromide fragments of collagen provided evidence that adhesion and activation of platelets by collagen was mediated through distinct receptors. These two events are mediated through distinct regions of the collagen molecule. It has been proposed that the activation of platelets by collagen occurs through a 'two-step' interaction, in which integrin $\alpha_2\beta_1$ provides an initial site of adhesion that brings a second site in the collagen molecule into the vicinity of a receptor responsible for activation.

6.4.4 Synthetic collagen peptide

The 22-amino-acid peptide, described in Chapter 4, was synthesised and its biological effects tested (Chapter 5). The peptide was tested for its ability to stimulate fibroblast chemotaxis in three different cell lines (L929, RWF, 3T3) using the Boyden Chamber Assay. The synthetic collagen peptide stimulated chemotaxis in all three fibroblast cell types, from which it may be concluded that fibroblasts have cell surface receptors which bind a region of this collagen peptide. However, the peptide was not significantly chemotactic for endothelial cells, implying that these cells have different cell surface receptors which do not recognise and therefore do not bind the collagen

peptide.

Known collagen peptide sequences which bind to cell surface receptors are D-G-E-A (Asp-Gly-Glu-Ala) and R-G-D (Arg-Gly-Asp) (Staatz *et al.*, 1991; Hynes 1992). In this work neither of these were identified as the collagen peptide recognition sequences. A new tetra-peptide Gly-Pro-Ala-Gly was identified as being recognised by fibroblast cell surface receptors during stimulation of chemotactic activity. This peptide is therefore likely to be the minimum amino acid sequence from Type I collagen which makes the most efficient contact with the appropriate receptor(s) on fibroblast membranes. It has been suggested that the chemotactic activity is unmasked as the size of the collagen-derived peptides decreases (Malone *et al.*, 1991). Previous work by Laskin *et al.* (1994), has shown the importance of Gly-Pro in stimulating macrophage migration: our work partly agrees with this finding (Gly-Pro are an essential part of an active peptide) but in this study it was found that shortening of the collagen-derived tetramer destroyed the chemotactic activity of the peptide towards fibroblasts. Perhaps macrophages and fibroblasts recognise similar but not identical amino acid sequences from collagen peptides.

6.4.5 Known cell-surface receptors

It would be of great importance to identify and isolate collagen-related cell surface receptors. However, there are already conflicting results on this matter. Many different putative collagen receptor molecules have been identified (Ruoslahti *et al.*, 1994), notably integrins, which are a group of cell surface molecules that mediate the attachment of cells to collagen. Integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$ are known to be involved in cell-collagen interactions although other non-integrin collagen receptors and collagen binding proteins have been documented (Akiyama *et al,* 1990). Integrin $\alpha 3\beta 1$ has been proposed as a collagen receptor (Elices *et al.*, 1991), however this is controversial (Gullberg *et al.*, 1992; Tuckwell *et al.*, 1996; Messent *et al.*, 1998).

It has been shown that a binding site for the integrin $\alpha 2\beta 1$ is located within the $\alpha 1$(I) CB3 fragment of collagen (Staatz *et al.*, 1990) and that the amino acid sequence Asp-Gly-Glu-Ala (D-G-E-A) is an important structural determinant of the $\alpha 2\beta 1$ recognition site (Staatz *et al.*, 1991). However, the importance of the D-G-E-A sequence as a structural determinant of the $\alpha 2\beta 1$ recognition site is now widely doubted, having been found to be inactive by other groups (Cardarelli *et al.*, 1992; Tuckwell *et al.*, 1996). Binding sites for $\alpha 1\beta 1$ have been shown to be present in both $\alpha 1$(I) CB3 and $\alpha 1$(I) CB8; $\alpha 1$(I) CB3 also contains a binding site for $\alpha 2\beta 1$ (Gullberg *et al.*, 1992). Recent work by Knight *et al.*, 2000, has identified an integrin $\alpha 2\beta 1$ binding site in $\alpha 1$(I) CB3 containing an essential G-E-R sequence. These findings point to the conclusion that Collagen I contains separate binding sites for $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins. The results in this thesis are in accordance with these findings.

6.4.6 Structural significance

The structure of peptide species, whether single-stranded or, triple-helical, is a relevant factor when considering their interactions with other extracellular matrix components (Rossi *et al.*, 1996). Conformation and length of a peptide are also extremely important in determining chemotactic potency. According to Malone *et al.*, (1991), chemotactic activity is unmasked as the size of the collagen derived peptide decreases. Larger peptides generated after digestion may interfere with receptor binding through steric hindrance (Laskin *et al.*, 1994). Steric hindrance is a term intended to denote the influence exerted in a reacting group by the spatial arrangement of neighbouring atoms. Substituting groups can prevent the approach to other groups by reason of their size (Daintith, 1990). A critical length may be required for maximal activity. Larger peptides may inhibit binding to the cell surface receptor and hence chemotactic activity will be blocked.

It has been suggested that conformation-dependent collagen binding sites may exist

(Gullberg *et al.*, 1992). The D-G-E-A binding site for the $\alpha 2\beta 1$ integrin was described as a single linear sequence (Santoro *et al.*, 1991). Tuckwell *et al.*, (1996), described the inability of CNBr fragments to reproduce the cell-binding of native collagen. This demonstrated a strict dependence on collagen conformation. Binding of denatured type I collagen has been postulated to be mediated by $\alpha_V\beta_3$ or $\alpha 5\beta_1$ integrins rather than $\alpha 2\beta$ integrin, or via a fibronectin bridge (Messent *et al.*, 1998). The identification of this bridge adds to the mechanisms by which cells can bind to denatured collagen. This work suggests that collagen-integrin binding requires an intact triple helix. There is the possibility that the CNBr peptides used in the work presented here could renature to the triple helical conformation. Circular dichroism measurements could reveal this. Since the time limitations of this study prevent further investigations into receptors and binding sites no conclusions on this can be made, but the complexity of this matter has already been pointed out. Work by Rossi *et al.*, 1996, has shown that the circular dichroism profile of CNBr peptides at increasing temperatures showed that the melting temperature for triple-helical peptides is about 6-10°C lower than that of the parent native Type I collagen, indicating that CNBr peptides are most likely to be non-triple helical.

6.5 *IN VIVO* WOUND HEALING STUDIES

Even though the collagen peptides were isolated *in vitro,* it is probable that the peptide occurs naturally in the tissue breakdown process. In the body, collagen molecules are cleaved by mammalian collagenase to give two triple helical fragments (Gross & Nagai 1965). Both fragments denature at physiological temperatures and are readily degraded by most proteases (Stark & Kuhn 1968). It is more than likely that a whole range of peptides are generated. Other molecules of the ECM can also be degraded and a variety of peptides with biological properties isolated: Elastin peptides (Fulop *et al.*, 1998), fibronectin fragments (Elices *et al.*, 1994), laminin peptides (Hershkaie *et al.*, 1995) and hyaluronic acid (Chen & Abatangelo 1999) all stimulate cellular migration. Again it is possible that the mechanisms resulting in the formation of ECM-derived bioactive peptides have the potential to take place naturally. It could be possible to examine wound fluids and tissues to look for the actual presence of these peptides. This has been done for instance in synovial fluid (from rheumatoid arthritis; Miyake *et al.*, 1993) and periodontal disease fluid(Layik *et al.*, 2000).

6.5.1 Animal Models

In order to provide backup and support to *in vitro* findings in many wound healing studies, an *in vivo* (animal) model may be used.

Many species of animal have been used to model the healing process in humans. Winter (1968) studied the role of occlusion using the domestic pig while Silver (1969) studied angiogenesis using the rabbit ear chamber model. Genetically modified mice have recently become an invaluable tool in wound healing. These knockout and transgenic mice could be used to study many areas of wound healing (Scheid *et al.*, 2000). Due to variations in morphology and physiology among animals, as well as among humans, it is difficult to have one model to suit all needs. Often the choice of an animal model is directed by practical issues such as cost, housing costs, and animal

husbandry (Davidson 1998). The fact that they are 'models' also shows that they are only trying to be representative of a process and hence not definite in showing how the wounding process actually functions. The model chosen in this study was the PVA sponge model (Coomes *et al.*, 1997; Shah *et al.*, 1999).

6.5.1.1 PVA sponge model

PVA (polyvinyl alcohol) sponges containing the synthetic collagen peptide were injected into rats and removed after 7 and 10 days post-implantation. The sponges were then analysed both biochemically and histologically. The DNA, protein and collagen content of collagen-containing sponges were compared with control (PBS only) sponges. Sections of sponge, stained with haematoxylin and eosin, were examined microscopically for fibrin and granulation tissue content. Again collagen sponges were compared to control sponges.

From the results in Chapter 5, it was found that PVA sponges containing the collagen peptide increased DNA, protein and collagen content of the test sponge compared to control sponges. Histologically, the collagen containing sponges were also observed to have increased fibrin and granulation tissue content compared to controls. Both of these effects were shown to be concentration-dependant.

The PVA sponge was used as a model of wound healing but it more accurately reflects the host response to a foreign body. The sponge is a defined volume which with time fills with various components, as does the wound itself. i.e. first a fibrin structure, then inflammatory cells, next fibroblasts, then the laying down and maturing of collagen structures and lastly apoptosis. In this study sponges were removed after 7 and 10 days, in order to measure collagen production and granulation tissue formation. However, sponges can be removed at other times, this will depend on which factors are being monitored. It should be noted that none of these timings are exact, as the response of the animals can vary with age, nutrition, etc. However, in

this model it was the comparison between control and collagen peptide injected sponges which is important.

The PVA sponge is a defined volume and provides a reliable method of collecting granulation tissue (Broadley *et al* 1989). The sponge itself is inert and does not interfere with any of the subsequent tests. Limitations of the choice model are that epithelial components cannot be measured. This is true, however, of all dead-space models of this type (stainless steel chambers, PTFE tubing; Davidson 1998). In the histological analysis, inflammatory cells (monocytes/macrophages) and fibroblasts cannot be distinguished between. A way this could be addressed for further study would be by differential staining.

6.6 CONCLUSIONS AND FUTURE WORK

It was a further object of this study to identify collagen peptides which are chemotactic at much lower concentrations than other known chemoattractants. The collagen tetra-peptide (Gly-Pro-Ala-Gly) elicited a maximal chemotactic response at 50ng/ml. In prior studies, Postlethwaite *et al.* 1978, found that collagen peptides induced maximal activity at a concentration of 900μg/ml. In 1985, Albini & Adelman-Grill found that collagen digested with mammalian collagenase stimulated fibroblast migration in the range of 25-50μg/ml. Cell derived and plasma fibronectin stimulate migration of fibroblasts in a dose-dependent manner, with maximal migration occurring at a concentration of 2μg/ml. Non-gelatin binding FN fragments are chemotactic at a concentration ranging from 2.5-50μg/ml, with maximal response at 10μg/ml (Postlethwaite *et al.,* 1981). Elastin-derived peptides also elicit chemotactic responses, maximal at 0.5-2.0μg protein/ml (Senior *et al.*, 1982). Growth factors are more potent chemoattractants for fibroblasts. PDGF gave maximal response at concentrations between 5-20ng/ml (Siegbaln *et al.*, 1990). FGF was maximal between 100-200ng/ml, EGF being maximal at 10-50ng/ml and TGF-β being the most potent chemoattractant at a concentration of 1pg/ml (Grant *et al.,* 1992). The peptide concentration (50ng/ml) of the collagen peptide identified in this study was of a similar range to many of these chemotactic growth factors. This peptide is therefore a potent chemoattractant and could be used in future studies on wound healing.

It must be noted that there has been very little recent work on collagen and its biological uses. This could be due to the fact that collagen peptides in prior studies were not potent enough and would not have much effect on wound healing especially compared to growth factors. Though of course scientific research does follow trends and fashions and it could be that growth factor research is a developing research area. Growth factors are easier to get, you can buy them commercially, whereas with many of the collagen types purification is a difficult and laborious process. Growth factors may also be easier to work with. However, even growth factors (Singer & Clark

1999) have not reached commercial success at this time. Therefore, the uses of this collagen peptide may also be limited.

Further work on identification and isolation of the cell surface receptors involved in these processes would be extremely useful. The work of Tuckwell *et al.* (1996), suggested that only triple helical collagen was involved in integrin-mediated receptor binding. Non-triple helical collagen peptides may use another non-integrin mediated receptor mechanism. The initial studies in this work appear to agree with the findings of Tuckwell *et al.*, in that the collagen peptides isolated in this work do not bind to integrin receptors on the fibroblasts.

The importance of *in vivo* work is that the findings could be translated into potential uses in speeding up wound healing. From this preliminary *in vivo* study, further studies using the collagen tetrapeptide may be carried out i.e. pig model system. This model is better matched chronologically and physiologically to the patient population (Davidson 1998). The eventual use of the peptide could be in topical application to a wound with the collagen peptide in a pharmaceutically acceptable carrier. Suitable carriers include: creams and ointments and hydrogels containing cellulose. The peptide could also be coated onto, or incorporated into a solid wound dressing such as film, or a fibrous pad or a sponge.

CHAPTER 7:

REFERENCES

Abercrombie M, Heaysman JE, Pogrum SM (1972) *The locomotion of fibroblasts in culture* Exp Cell Res 73:536-539

Adzick NS, Longaker MT (1992) *Scarless fetal healing* Ann Surg 215:3-7

Adzick NS, Lorenz HP (1994) *Cells, matrix, growth factors and the surgeon. The biology of scarless fetal wound repair* Ann Surg 220:10-18

Akiyama SK, Nagata K, Yamada KM (1990) *Cell surface receptors for extracellular matrix components* Biochim Biophs Acta 1031:91-110

Alberio L, Dale GL (1999) *Platelet-collagen interactions: membrane receptors and intracellular signalling pathways* Eur J Clin Invest 29:1066-76

Albini A, Richter H, Pontz BF (1983) *Localisation of the chemotactic domain in fibronectin* FEBS Lett 156:222-226

Albini A, Adelmann-Grill BC (1985) *Collagenolytic cleavage products of collagen type I as chemoattractants for human dermal fibroblasts* Eur J Cell Biol 36:104-107

Altman DG ed (1994) Practical statistics for medical researchers Chapman & Hall: London

Anderson SML, Elliott RJ (1991) *Evaluation of a new, rapid collagen assay* Biochem Soc Trans 19:389S

Anderson (1992) In :Pathological basis of the connective tissue disease Gardner DL, ed. Chp 4 London :Edward Arnold

Andriessen MP, van der Born J, Latijnhouwers, Bergers M, van der Kerkhof PC, Schalkwijk J (1997) *Basal membrane heparan sulphate proteoglycan expression during wound healing in human skin* J Pathol 183:264-271

Aspenstrom P (1999) *The Rho GTPases have multiple effects on the actin cytoskeleton* Exp Cell Res 246:20-25

Asselin J, Gibbins JM, Achison M, Lee YH, Morton LF, Farndale RW, Barnes MJ, Watson SP (1997) *A collagen-like peptide stimulates tyrosine phosphorylation of syk and phospholipase Cγ2 platelets independent of the integrin α2β1* Blood 89:1235-1242

Aumailley M, Nurcombe V, Edgar D, Paulsson M, Timple R (1987) *The cellular interactions of laminin fragments. Cell adhesion correlates with two fragment-specific high affinity binding sites* J Biol Chem 262:11532-11538

Avraham S, Stevens RL, Nicodemuss CF, Gartner MC, Austen KF, Weiss JH (1989) *Molecular cloning of a cDNA that encodes the peptide core of a mouse mast cell secretory granule proteoglycan and comparison with analogous rat and human cDNA* Proc Nat Acad Sci USA 86:3763-3676

Ayad S, Boot-Handford RP, Humphries MJ, Kadler KE, Shuttleworth CA, eds. (1994) In :The Extracellular Matrix Facts Book London :Academic Press

Bailey AJ, Light ND, eds. (1989) In :Connective Tissue in meat and meat products London: Elsevier Applied Science

Barber TA, Ihlenfeld JV, Mathis TR, Mosher DF, Riddle LM, Hart AP, Updike ST, Cooper SL (1978) *Transient in vivo thrombus deposition onto polymeric biomaterials: role of plasma fibronectin* Trans Am Soc Artif Intern Organs 24:727-735

Barnes MJ (1982) *The collagen-platelet reaction* In: Collagen in health and disease Weiss JB, Jayson MIV Eds p179-197 Churchill Livingston: Edinburgh

Barnes MJ, Knight CG, Farndale RW (1996) *The use of collagen-based model peptides to investigate platelet-reactive sequences in collagen* Biopolymers 40:383-397

Barrett AJ (1990) *The classification of proteinases* In: Protein Degradation in Health and Disease Ciba Found Sym 75:1-13

Bateman JF, Mascara T, Chan D, Cole WG (1986) *Rapid fractionation of collagen chains and peptides by high performance liquid chromatography* Anal Biochem 154:338-344

Baum JL (1971) *Source of the fibroblast in the central corneal wound healing* Arch Ophthalmol 85:473-477

Becker S (1988) *Functions of the human mononuclear phagocyte system* Adv Drug Deliv Res 2:1-12

Bennett NT, Schultz GS (1993) *Growth factors and wound healing: Biochemical properties of growth factors and their receptors* Am J Surg 165:728-737

Bevilacqua MP, Amrani D, Mosesson MW, Bianco C (1981) *Receptors for cold-insoluble globulin (plasma fibronectin) on human monocytes* J Exp Med 153:42-60

Berg RA (1982) *Determination of 3- and 4-hydroxyproline* Meth Enzymol 82:372-378

Birkedale-Hansen H, Moore WG, Bodden MK, Windsor LJ, Birkedale-Hansen B, DeCarlo A, Engler JA (1993) *Matrix metalloproteinases: a review* Crit Rev Oral Biol Med 4:197-250

Bisaccia F, Castiglione Morelli MA, De Biasi M, Traniellos S, Spisani S, Tamburro AM (1994) *Migration of monocytes in the presence of elastolytic fragments of elastin and in synthetic derivatives* Int J Peptide Protein Res 44:332-341

Bishop JE, Laurent GJ (1995) *Collagen turnover and its regulation in the normal and hypertrophying heart* Eur Heart J 16 (Suppl C):38-44

Bleiberg I, Harvey AK, Smale G, Grotendorst GR (1985) *Identification of a PDGF-like mitoattractant produced by NIH/3T3 cells after transformation with SV40* J Cell Physiol 123:161-166

Bond MD, Van Wart HE (1984) *Relationship between the individual collagenases of Clostridium histolyticum: evidence for evolution of gene duplication* Biochem 23:3092-3099

Booyse FM, Sedlak BJ, Rafelson ME (1975) *Culture of arterial endothelial cells: characterisation and growth of bovine aortic cells* Thromb Diath Haemorrh 34:825-839

Booyse FM, Feder S, Quarfoot AJ (1982) *Culture-produced subendothelium. II. Effect of plasma, F.VIII.R.WF and fibronectin on interaction of normal platelets with normal and von Willebrand porcine aortic subendothelium* Thromb Res 28:299-311

Bowersox JC, Sorgente N (1982) *Chemotaxis of aortic endothelial cells in response to fibronectin* Cancer Res 42:2547-2551

Boyden S (1962) *Chemotactic effect of mixtures of antibody and antigen on polymorphonuclear leukocytes* J Exp Med 115:453-466

Braddock M, Campbell CJ, Zuder D (1999) *Current therapies for wound healing: electrical stimulation, biological therapeutics, and potential for gene therapy* Int J Dermatol 38:808-17

Bradford MM (1976) *A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding Anal Biochem* 72:248-254

Brennan FR, Jones TD, Longstaff M, Chapman S, Bellaby T, Smith H, Xu F, Hamilton WDO, Flock JI (1999) *Immunogenicity of peptides derived from a fibronectin-binding protein of S. aureus expressed on two different plant viruses* Vaccine 17:1846-1857

Broadley KN, Aquino AM, Hicks B, Ditesheim JA, McGee GS, Demetriou AA, Woodward SC, Davidson JM (1989) *The diabetic rate as an impaired wound healing model: stimulatory effects of transforming growth factor-beta and basic fibroblast growth factor* Biotechnol Ther 1:55-68

Brockhausen I, Schutzbach J, Kuhn W (1998) *Glycoproteins and their relationship to human disease* Acta Anat 161:36-78

Brown JC, Golbik R, Mann K, Timple R (1994) *Structure and stability of the triple-helical domains of human collagen XIV* Matrix Biology 14:287-295

Brownell AG, Bessem CC, Slavkin HC (1981) *Possible functions of mesenchyme cell-derived fibronectin during formation of basal lamina* Proc Nat Acad Sci USA 78:3711-3715

Bruijn JA, Roo A, de Geus B, de Heer E (1994) *Transforming growth factor–β and the glomerular ECM in renal pathology* J Clin Med 123:34-47

Burgeson RE (1988) *New collagens, new concepts* Ann Rev Cell Biol 4:551-577

Burgeson RE (1993) *Type VII collagen, anchoring fibrils and epidermolysis bullosa* J Invest Dermatol 101:252-255

Burke JM, Ross R (1979) *Synthesis of connective tissue macromolecules by smooth muscle* In Rev Conn Tiss Res 8:119-157

Burton K (1956) *A study of the conditions and mechanisms of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid* Biochem J 62:315-323

Busby JF, Argravesw S, Brew SA, Pechnik I, Gilliland GL, Ingham KC (1995) *Heparin binding by fibronectin module III-13 involves six discontinuous basic residues brought together to form a cationic cradle* J Biol Chem 270:18558-18562

Buttle DJ, Handley CJ, Ilic M, Saklatvala J, Murata M, Barrett AJ (1993) *Inhibition of cartilage proteoglycan release by a specific inactivator of cathepsin B and an inhibitor of matrix metalloproteinases* Arthritis & Rheumatism 36:1709-1717

Cantor JO, Keller S, Parshley MS, Darnule TV, Darnule AT, Cerreta JM, Turino GM, Mandle I (1980) *Synthesis of crosslinked elastin by an endothelial cell culture* Biochem Biophys Res Comm 95:1381-1386

Cardarelli PM, Yamagata S, Taguchi I, Gorcsan F, Chiang SL, Lobl T (1992) *The collagen receptor $\alpha_2\beta_1$ from MG-63 and HT 1080 cells interacts with a cyclic RGD peptide* J Biol Chem 267:23159-23164

Carpino LA, Han GY (1972) *The 9-fluorenylmethoxycarbonyl amino-protecting group* J Org Chem 3404-3409

Carsons SE, ed (1990) In: Fibronectin in health and disease Boca Raton, Fla CRC Press

Caterina MJ, Devreotes PN (1991) *Molecular insights into eukaryotic chemotaxis* FASEB J 5:3078-3085

Cawston TE (1996) *Metalloproteinase inhibitors and the prevention of connective tissue breakdown* Pharmacol Ther 70:163-182

Chen JWY, Abatangelo G (1999) *Functions of hyaluronan in wound repair* Wound Rep Reg 7:79-89

Cheresh DA (1991) *Integrins in thrombosis, wound healing and cancer* Biochem Soc Trans 19:835-838

Chothia C, Jones EY (1997) *The molecular structure of cell adhesion molecules* Ann Rev Biochem 66:823-62

Chvapil M (1977) *Reconstituted collagen* In :Biology of collagen Viidik & Vuust, eds. London :Academic Press

Chvapil M (1982) *Considerations on manufacturing principles of a synthetic burn dress* J Biomed Mat Res 16:245-263

Chvapil M, Chvapil JA, Owen JA (1986) *Reaction of various skin wounds in the rat to collagen sponge dressings* J Surg Res 41:410-418

Clark RAF (1985) *Cutaneous tissue repair: basic biological considerations* J Am Acad Dermatol 13:701-725

Clark RAF (1993) *Biology of dermal wound care* Dermatol Clin 11:647-666

Clark RAF (1996) In :The Molecular and Cellular Biology of Wound Repair. Clark RAF & Henson PM, eds. NY : Plenum Press

Cohen K, Moore CD, Diegelmann RF (1979) *Onset and localisation of collagen synthesis during wound healing in open rat skin wounds* Proc Soc Exp Biol Med 160:458-465

Cooney R, Iocono J, Maish G, Stanley-Smith J, Ehrlich P (1997) *Tumor necrosis factor mediates impaired wound healing in chronic abdominal sepsis* J Trauma 42:415-420

Cucin RL, Barek D (1983) *Complications of injectable implants* Plast Reconst Surg 71:731-736

Daintith A ed. (1990) A concise dictionary of chemistry Oxford: Oxford University Press

Davidson JM (1998) *Animal models for wound repair* Arch Dermatol Res 290:S1-S11

Davies JS, Enjalbal C, Wise C, Jones G, Handa BK (1994) *Further studies in cyclic RGD analogues* Biochem Soc Trans 22:1049-1052

Davis GE (1992) *Affinity of integrins for damaged extracellular matrix: $\alpha_V\beta_3$ binds to denatured collagen types I through RGD sites* Biochem Biophys Res Commun 182:1025-1031

Delustro F, Smith ST, Sundsmo J, Salem G, Kincaid S, Ellingsworth L (1987) *Reaction to injectable collagen: results in animal models and clinical use* Plast Reconst Surg 79:581-594

Denhardt DT, Feng B, Edwards DR, Cocuzzi ET, Malyanlar UM (1993) *Tissue inhibitor of metalloproteinases - structure, control of expression and biological functions* Pharmacol Ther 59:329-341

Desmouliere A, Redard M, Darby I, Gabbiani G (1995) *Apoptosis mediated the decrease in cellularity during the transition between granulation tissue and scar* Am J Pathol 146:56-66

Doanati L, Magliano E, Colonna M, Garbin S (1994) *Signal versus enzymatic debridement* In :Proteolytic Enzymes and Wound Healing Westerhof & Vanscheidt, eds.p31-47 NY: Plenum Press

Doillon CJ, Silver FH, Berg RA (1987) *Fibroblast growth on a porous collagen sponge containing hyaluronic acid and fibronectin* Biomaterials 8:195-200

Doillon CJ, Silver FH, Olsen RA, Chandrakala Y, Berg RA (1988) *Fibroblast and epidermal cell type I collagen interactions* Scan Micro 2:985-992

Donaldson DJ, Mahan JT (1983) *Fibrinogen and fibronectin as substrates for epidermal cell migration during wound closure* J Cell Sci 62:117-127

Donnelly J (1998) *Wound healing-from poultices to maggots (a short synopsis of wound healing through the ages* Ulster Med J 67:S1 47-51

Downey GP (1994) *Mechanisms of leukocyte motility and chemotaxis* Curr Opin Immun 6:113-124

Drake Sl, Klein DJ, Mickelson DJ, Oegema TR, Furcht LT, McCarthy JB (1992) *Cell surface phosphatidylinositol-anchored heparan sulphate proteoglycan initiates mouse melanoma cell adhesion to a fibronectin-derived heparin-binding synthetic peptide* J Cell Biol 117:1331-1341

Dufour S, Duband JL, Humphries MJ, Obara M, Yamada KM, Thiery JD (1988) *Attachment, spreading and locomotion of avian neural crest cells are mediated by multiple adhesion sites on fibronectin molecules* EMBO J 7:2661-2671

Dziadek M (1995) *Role of laminin-nidogen complexes in basement membrane formation during embryonic development* Experientia 51:901-913

Ekblom M, Falk M, Salmivirta K, Durbeej M, Ekblom P (1998) *Laminin isoforms and epithelial development* Ann NY Acad Sci 857:194-211

Elices MJ, Urry LA, Hemler ME (1991) *Receptor functions of the integrin VLA-3: fibronectin, collagen & laminin binding are differentially influenced by Arg-Gly-Asp peptide and by divalent cations* J Cell Biol 112:169-181

Elices MJ, Tsai V, Strahl D, Goel AS, Tollefson V, Arrhenius T, Wayner EA, Gaeta FCA, Fikes JD, Firestein GS (1994) *Expression and functional significance of alternatively spliced CSI fibronectin in rheumatoid arthritis microvasculature* J Clin Invest 93:405-416

Engel J (1991) *Common structural motifs in proteins of the extracellular matrix* Curr Opin Cell Biol 3:779-785

Engel J (1992) *Laminins and other strange proteins* Biochem 31:10643-10651

Esko JD (1991) *Genetic analysis of proteoglycan structure, function and metabolism* Curr Opin Cell Biol 3:805-816

Falk W, Goodwin RH, Leonard EJ (1980) *A 48-well chemotaxis assembly for rapid and accurate measurement of leukocyte migration* J Immunol Meth 33:239-247

Ferguson MW (1998) *Wound healing - scar wars* Ulster Med J 67 S1:37-40

Fields GB (1991) *A model for interstitial collagen catabolism by mammalian collagenases* J Theor Biol 153:585-602

Finesmith TH, Broadley KN, Davidson JM (1990) *Fibroblasts from wounds of different stages of repair vary in their ability to contract a collagen gel in response to growth factors* J Cell Physiol 44:99-107

Fitzsimmons CM, Cockburn CG, Hornsey V, Prowse CV, Barnes MJ (1988) *The interaction of von Willebrand factor (VWF) with collagen: investigation of VWF binding sites in the collagen molecule* Thrombosis & Haemostasis 59:186-192

Fleischmajer R, Fisher LW, MacDonald ED, Jacobs L, Perlish JS, Termine JD (1991) *Decorin interacts with fibrillar collagen of embryonic and adult human skin* J Struc Biol 106:82-96

Fleischmajer R, Olsen BR, Kuhn K (1990) *Structure, molecular biology and pathology of collagen* Ann NY Acad Sci 580:1-592

Forget MA, Desrosiers RR, Beliveau R (1999) *Physiological roles of matrix metalloproteinases: implications for tumor growth and metastasis* Can J Physiol Pharmacol 77:465-80

Freshney RI, ed. (2000) In: Culture of animal cells: a manual of basic technique, NY, Wiley-Liss 3rd Edition

Frishman WH, Burns B, Atac B, Alturk N, Altajar B, Lerrick K (1995) *Novel antiplatelet therapies for treatment of patients with ischemic heart disease: inhibitors of the platelet glycoprotein integrin receptor* Am Heart J 130:887-892

Fukuda K, Koshihara Y, Oda H, Ohyama M, Ooyama T (1988) Type V *collagen selectively inhibits human endothelial cell proliferation* Biochem Biophys Res Comm 151:1060-1068

Fulop T, Jacob MP, Khalil A, Wallach J, Robert L (1998) *Biological effects of elastin peptides* Path Biol 46:496-506

Gabbiani G (1992) *The biology of the myofibroblast* Kid Int 41:530-532

Gabka CJ, Benhaim D, Mathes S, Scheuenstuhl H, Chan A, Fu KK, Hunt TK (1995) *An experimental mode to determine the effect of irradiated tissue on neutrophil function* Plas Recon Surg 96:1676-1688

Gailit J, Clark RAF (1994) *Wound repair in the context of the extracellular matrix* Curr Opin Cell Biol 6:717-725

Garfin DE (1990) *One dimensional gel electrophoresis* Meth Enzymol 182:425-441

Gendimenco GJ, Bouquin PL, Tramposch KM (1988) *Diphenylamine colorimetric method for DNA assay: a shortened procedure by incubating samples at 56℃* Anal Biochem 173:45-48

Gilchrest BA, Calhoun JK, Maciag T (1982) *Attachment and growth of human keratinocytes in a serum-free environment* J Cell Physiol 112:197-206

Ginsberg MH, Painter RG, Birdwell C, Plow EF, Forsyth J (1979) *The detection, immunofluorescent localisation, and thrombin induced release of human platelet-associated fibronectin antigen* J Supramol Struc 11:167-174

Gleiber WE, Schifmann E (1984) *Identification of a chemoattractant for fibroblasts produced by human breast carcinoma lines* Cancer Res 44:3398-402

Goslen JB (1988) *Wound healing for the dermatological surgeon* Dermatol Surg Oncol 14:959-972

Grant MB, Khaw PT, Schultz GS, Adams JL, Shimizu RW (1992) *Effects of epidermal growth factor, fibroblast growth factor and transforming growth factor-beta on corneal cell chemotaxis* Invest Ophthalmol Vis Sci 33:3292-3301

Green LJ, Mould AP, Humphries MJ (1998) *The integrin β subunit* Int J Biochem Cell Biol 30:179-184

Greenwald RA, Woessner JR (1999) *Common names of matrix metalloproteinases* Ann NY Acad Sci 878 pXIX

Grinnell F (1978) *Cellular adhesiveness and extracellular substrata* Int Rev Cytol 53:65-144

Gross E (1967) *The cyanogen bromide reaction* Methods Enzymol 11:238-240

Gross J, Nagai (1965) *Specific degradation of the collagen molecule by tadpole collagenolytic enzyme* Proc Nat Acad Sci USA 54:1197-1204

Gross L, Lapiere CM (1962) *Collagenolytic activity in amphibian tissues. A tissue culture assay* Proc Nat Acad Sci USA 48:1014-1022

Grosso LE, Scott (1993) *PGAIPG, a repeated hexapeptide of bovine tropoelastin, is a ligand for the 67kDa bovine elastin receptor* Matrix 13:157-164

Grosso LE, Scott M (1993) *PGAIPG, a repeated hexapeptide of bovine and human tropoelastin, is chemotactic for neutrophils and Lewis Lung carcinoma cells* Arch Biochem Biophys 305:401-404

Grotendorst GR (1984) Structural and regulatory components of wound healing. Chemoattractants and growth factors In: Wound Repair Peacock EE ed Philadelphia: Saunders p237-246

Gullberg D, Gehlsen KR, Turner DC, Ahlenk K, Zijenah LS, Barnes MT, Rubin K (1992) *Analysis of $\alpha_1\beta_1$, $\alpha_2\beta_1$ and $\alpha_3\beta_1$ integrins in cell-collagen interactions: identification of conformation dependent $\alpha_3\beta_1$ binding sites in collagen type I* EMBO J 11:3865-3873

Han J, Ohno N, Pasco S, Monboisse J, Borel JP, Kefalides NA (1997) *A cell binding domain from the $\alpha 3$ chain of Type IV collagen inhibits proliferation of melanoma cells* J Biol Chem 272:20395-20401

Harcharan KR, McCormick D (1997) *Proteases and their inhibitors in human brain tumours : a review* Anticancer Res 17:4151-4162

Hardingham TE, Fosang AJ (1992) *Proteoglycans: many forms and many functions* FASEB J 6:861-870

Harper E (1980) *Collagenases* Ann Rev Biochem 49:1063-1078

Harrington (1996) *Bacterial collagenases and collagen-degrading enzymes and their potential role in human disease* Infection Imm 104:1885-1891

Harris A, Dunn G (1972) *Centripetal transport of attached peptides on both surfaces of moving fibroblasts* Exp Cell Res 73:519-523

Hatz RA, von Jan NCS, Schildberg FW (1994) *The role of collagenase in wound healing* In :Proteolytic enzymes. Westerhof & Vanscheidt, eds. p75-87 Oxford:IRL Press

Hay ED, ed (1991) In :Cell Biology of the ECM chp 12 p396-400 London: Plenum Press

Hayes PH, Scott GK, Ng NF, Hew CL, Davies PL (1989) *Cysteine-rich type II antifreeze protein precursor is initiated from the third AUG codon of its mRNA* J Biol Chem 264:18761-18767

Hedman K, Johansson S, Vartio T, Kjellen L, Vaheri A, Hook M (1982) *Structure of the pericellular matrix: association of heparin and chondroitin sulfates with fibronectin-procollagen fibers* Cell 28:663-671

Heinegard D, Franzen A, Hedbam E, Sommarin Y (1986) *Common structures of the core proteins of interstitial proteoglycans* Ciba Foundation Symp 124:69-88

Heldin CH, Westermark B (1999) *Mechanism of action and in vivo role of platelet-derived growth factor* Physiol Rev 79:1283-316

Hemler ME (1999) In :Guidebook to the ECM, anchor and adhesion proteins Kreis J, Vale R, eds. p196-212 Oxford University Press

Hershkoviz R, Goldkorn I, Lider O (1995) *Tumour necrosis factor-α interacts with laminin and functions as a pro-adhesive cytokine* Immunology 85:125-130

Highberger JH, Corbett C, Gross J (1979) *Isolation and characterisation of a peptide containing the site of cleavage of the chick skin collagen α1(I) chain by animal collagenase* Biochem Biophy Res Commun 89:202-208

Hogaboam CM, Smith RE, Kunkel SL (1998) *Dynamic interactions between lung fibroblasts and leukocytes: implications for fibrotic lung disease* Proc Assoc Am Physicians 110:313-320

Hogaboam CM, Steinhauser ML, Chensue SW, Kunkel S (1998) New roles for chemokines and fibroblasts in interstitial fibrosis Kidney Int 54:2152-2159

Hood DB, Gettins, Johnson DA (1993) *Nitrogen dioxide reactivity with proteins: effects on activity and immunoreactivity with α-1-protease inhibitor and implications for NO_2-mediated peptide degradation* Arch Biochem Biophys 304:17-26

Hopkinson-Woolley J, Hughes D, Gordon S, Martin P (1994) *Macrophages recruitment during limb development and wound healing in embryonic and foetal mouse* J Cell Sci 107:1159-1167

Houck J, Chang C (1971) *The chemotactic properties of the products of collagenolysis* PSEBM 138:69-75

Hulmes DJ (1992) *The collagen superfamily - diverse structure and assemblies* Essays Biochem 27:49-67

Hunninghake GW, Davidson JM, Rennard S, Szapiel S, Gadek JE, Crystal RG (1981) *Elastin fragments attract macrophage precursors to diseased sites in pulmonary emphysema* Science 212:925-927

Hunt TK, La Van FB (1989) *Enhancement of wound healing by growth factors* N Eng J Med 321:111-112

Hynes RO (1989) *Alternative splicing of fibronectin is temporally and spatially regulated in the chick embryo* Development 106:375-388

Hynes RO (1992) *Integrins: versatility, modulation and signalling* Cell 69:11-25

Hynes RO (1999) In: Guidebook to the ECM, anchor and adhesion proteins Kreis T, Vale R, eds. p 422-425 Oxford University Press

Ihrcke NS, Wrenshall LE, Lindman BJ, Platt JL (1993) *Role of heparan sulfate in immune system-blood vessel interactions* Immunol Today 14:500-505

Ikeda M, Sorimachi K, Akimoto K, Yasumura Y (1993) *Reversed-phase HPLC analysis of hyp and pro from collagen by derivitisation with dansyl chloride* J Chrom 621:133-138

Indik Z, Yeh H, Ornstein-Goldstein N, Sheppard P, Anderson N, Rosenbloom JC, Peltonen L, Rosenbloom J (1987) *Alternative splicing of human elastin mRNA by sequence analysis of cloned genomic and complementary DNA* Proc Nat Acad Sci USA 84:5680-5684

Ingeber DE (1991) *Control of capillary growth and differentiation by ECM. Use of tensegrity (tensional integrity) mechanisms for signal processing* Chest 99 S3:34-40

Iozzo RV (1998) *Matrix Proteoglycans: from molecular design to cellular function* Ann Rev Biochem 67:609-652

Iozzo RV, Murdoch AD (1996) *Proteoglycans of the extracellular environment: clues from the gene and protein side offer novel perspectives in molecular diversity and function* FASEB J 10:598-614

Iredale JP (1997) *Tissue inhibitors of metalloproteinases in liver fibrosis* Int J Biochem Cell Biol 29:43-54

Irvin TT, ed. (1981) In: Wound Healing, Principles and Practice p1-34 London :Chapman & Hall

Jarstrand C, Ahlgren T, Berghen L (1982) *Fibronectin increases the motility, phagocytosis and NBT (nitroblue tetrazolium) - reduction of granulocytes* J Clin Lab Imm 8:59-63

Johnston DE (1979) *The healing process in open skin wounds* Comp Contin Educ 1:789-800

Johnston DE (1990) *Wound healing in skin* Vet Clin N Am 20:1-25

Juliano RL, Haskill S (1993) *Signal transduction from the extracellular matrix* J Cell Biol 120:577-585

Juliano RL, Varner JA (1993) *Adhesion molecules in cancer: the role of integrins* Curr Opin Cell Biol 5:812-816

Katz AR, Turner RJ (1970) *Evaluation of tensile and adsorption properties of polyglycolic acid sutures* Surg Gynecol Obstel 134:701-716

Keil B (1988) *Recent development in the research of structure-function relationships of collagenases* Pathol Biol 36:1112-1118

Keilty CM, Hopkinson J, Grant ME (1993) *Collagen: The collagen family: structure, assembly, and organisation in the extracellular matrix* In :Connective tissue and its heritable disorders. Molecular genetics and medical aspects Royce PH, Steinmann B, eds. p103-147 NY, Wiley-Liss

Kim WM, Kang K (2000) *Enzymatic and molecular biochemical characterisations of human neutrophil elastases and cathepsin G-like enzyme* Mol Cells 5:498-504

Kjellen L, Lindahl U (1991) *Proteoglycans: structure and interactions Ann Rev Biochem* 60:443-475

Klein TE & Huang CC (1999) *Computational investigations of structural changes resulting from point mutations in a collagen-like peptide* Biopolymers 47:167-184

Kleinmann HK, Kebe RJ, Martin GR (1981) *Role of collagenous matrices in the adhesion and growth of cells* J Cell Biol 88:473-485

Knight CG, Morton LF, Onley DJ, Peachy AR, Messent AJ, Smethurst PA, Tuckwell DS, Farndale RW, Barnes MJ (1998) *Identification in collagen type 1 of an integrin $\alpha_2\beta_1$-binding site containing an essential GER sequence* J Biol Chem 273:33287-33294

Knight CG, Morton LF, Onley DJ, Peachy AR, Ichinohe T, Okuma M, Farndale RW, Barnes MJ (1999) *Collagen-platelet interaction: Gly-Pro-Hyp is uniquely specific for platelet GPVI and mediates platelet activation by collagen* Cardiovascular Res 41:450-457

Knight CG, Morton LF, Peachey AR, Tuckwell DS, Farndale RW, Barnes MJ (2000) *The collagen-binding A-domain of integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$ recognise the same specific amino acid sequence, GFOGER in native (triple-helical) collagens* J Biol Chem 275:35-40

Knighton DR, Fiegel VD (1989) *The macrophages: Effector cell in wound repair* Prog Clin Biol Res 299:217-226

Kobata A (1992) *Structures and functions of the sugar chains of glycoproteins* Eur J Biochem 209:483-501

Kobayashi H, Terao T (1997) *Hyaluronan acid-specific regulation of cytokines by human uterine fibroblasts* Am J Physiol 276:C1151-9

Krawczyk WS (1971) *The pattern of epidermal cell migration during wound healing* J Cell Biol 49:247-263

Kresse H, Hausser H, Schonherr E, Bittner K (1994) *Biosynthesis and interactions of small Chondroitin Dermatan sulphate proteoglycans* Eur J Clin Chem Clin Biochem 32:259-264

Kuettner KE, Kimura JH (1985) *Proteoglycans: an overview* J Cell Biochem 27:327-336

Kuhn K (1986) *The collagen family - variations in the molecular and supermolecular structure* in "Connective tissue in normal and pathological status" Eds. Krieg T, Kuhn K Rheumatology 10:29-43

Kuhn K (1987) *The classical collagen types I, II and III* In :Structure and function of collagen types Mayne R, Burgeson RE, eds. p1-42 Oxford University Press

Kumar S, Kumar P, Pontin JM, Sattar A, Rooney P, Pye D, Hunter RD (1992) *Hyaluronic acid promotes and inhibits angiogenesis* In :Angiogenesis in health and disease Maragoudakis ME, ed.. p253-263 NY :Plenum Press

Kunitomo M, Jay M (1985) *Elastin fragment-induced monocyte chemotaxis, the role of desmosines* Inflammation 9:183-188

Labat-Robert J, Bihari-Varga M, Robert L (1990) *Extracellular Matrix* FEBS 268:386-393

Lackie JM, ed. (1986) *Chemotaxis* In :Cell Movement and cell behaviour Chpt 9 p220-251 London :Allen & Unwin

Laemmli UK (1970) *Cleavage of structural proteins during the assembly of the head of bacteriophage T4* Nature 227:680-685

Lamme EN, van Leeuwen RTJ, Brandsma K, Marle JV, Middelkoop E (2000) *Higher numbers of autologous fibroblasts in an artificial dermal substitute improve tissue regeneration and modulate scar tissue formation* J Pathol 190:595-603

Landeau J, Pellat B, Hornebeck W (1994) *Increased secretion of latent activity following contact between human skin fibroblasts and elastin derived peptides* Cell Biol Int 18:111-117

Lander AD (1999) In :Guidebook to the ECM, anchor and adhesion proteins Kreis J, Vale R, eds. p351-356 Oxford University Press

Lapiere J, Chen JD, Iwasaki T, Hu L, Uitto J, Woodley DT (1994) *Type VII collagen specifically binds fibronectin via a unique subdomain within the collagenous triple helix* J Invest Derm 103:637-641

Laskin DL, Soltys RA, Berg RA, Riley DJ (1994) *Activation of alveolar macrophages by native and synthetic collagen-like polypeptides* Am J Resp Cell Mol Biol 10:58-64

Laskin DL, Kimura T, Sakakibara S, Riley DJ, Berg RA (1986) *Chemotactic activity of collagen-like polypeptides for human peripheral blood monocytes* J Leuk Biol 39:255-266

Layik M, Yamalik N, Caglayan F, Kilinc K, Etikan I, Eratalay K (2000) Analysis of human gingival tissue and gingival crevicular fluid beta-glucuronidase activity in specific periodontal disease J Periodontal 4:618-624

Levitsky VY, Melik-Nubarov NS, Siksnis VA, Grinberg VY, Burova TV, Levashov AV, Mozhaev VV (1994) *Reversible conformational transition gives rise to 'zig-zag' temperature dependence of the rate constant of irreversible thermoinactivation of enzymes* Eur J Biochem 1994:219-230

Li C, McCarthy JB, Furcht LT, Fields GB (1997) *An all D-amino acid peptide model of α1(IV) 531-534 from Type IV collagen binds the $\alpha_3\beta_1$ integrin and mediates tumour cell adhesion, spreading and motility* Biochem 36:15404-15410

Lipowitz AJ (1985) *Tissue generation* In :Textbook of Small Animal Surgery Slatter DH, ed. Vol 1:22-29 Saunders Company Ltd: Philadelphia

Long MM, King VJ, Prasad KU, Freeman BA, Urry DW (1989) *Elastin repeat peptides as chemoattractants for bovine aortic endothelial cells* J Cell Physiol 140:512-518

Longaker MT, Adzick NS (1991) *The biology of fetal wound healing: A review* Plas Recon Surg 87:788-798

Lund PK (1994) *Insulin-like growth factor 1: Molecular biology and relevance to tissue-specific expression and action* Recent Prog Horm Res 49:125-148

Macarak EJ, Howard PS (1983) *Adhesion of endothelial cells to extracellular matrix proteins* J Cell Physiol 116:76-86

MacNeil S (1994) *What role does the ECM serve in skin grafting and wound healing?* Burns S67-70

Magarit C, Ibanez VM, Lloret J, Ventura H, Broto J, Blanco J, Isnard R, Allende H (1987) *Use of fibrin sealant and collagen as haemostatic agents* Trans Proc 19:3835-7

Majno G (1975) *The Healing Hand* In :Man and Wound in the Ancient World Harvard

Majno G, Joris I, eds. (1996) In :Cell, tissue and disease. Principles and general pathology Chp 14 p465-485 Blackwell Science: Oxford

Malinda KM, Kleinman HK (1996) *The Laminins* Int J Biochem Cell Biol 28:957-959

Malone JD, Richards M, Jeffrey JJ (1991) *Recruitment of peripheral mononuclear cells by mammalian collagenase digests of type I collagen* Matrix 11:289-295

Mandl F (1953) Heilanasthesie, Wien. Klin. Wchnschr 65:117

Mani R, Falanga V, Shearman CP, Sandeman D, eds. (1999) In :Chronic wound healing: clinical measurement and basic science WB Saunders Company Ltd: Philadelphia

Martin BM, Gimbrane MA Jr, Majeau GR, Unanue ER, Contran RS (1983) *Stimulation of human monocyte/macrophage-derived growth factor (MDGF) production by plasma fibronectin* Am J Pathol 111:367-373

Martin P (1997) *Wound Healing: aiming for perfect skin regeneration* Science 276:75-81

Mast BA, Haynes JH, Krummel TM, Diegelmann RF, Cohen IK (1994) *In vivo degradation of fetal wound HA results in increased fibroplasia, collagen deposition and neovascularisation* Plas R Surg 89:503-509

Matrisian LM (1992) *Matrix-degrading metalloproteinases* Bioessays 14:455-463

Matton G, Anseevw A, Dekeyser F (1985) *The history of injectable biomaterials and the biology of collagen* Aesth Plas Surg 9:133-140

Mayne R, Brewton RG (1993) *New members of the collagen superfamily* Curr Opin Cell Biol 5:883-890

McCarthy JB, Furcht LT (1984) *Laminin and fibronectin promote haptotactic migration of B16 mouse melanoma in vitro* J Cell Biol 98:1474-1478

McGrath MH, Hundahl SA (1982) *The spatial and temporal qualification of myofibroblasts* Plast Recon Surg 69:975-985

McGrath JA, Eady RA (1997) *Heparan Sulphate proteoglycan and wound healing in skin* J Pathol 183:251-255

McMinn RM (1967) *The cellular morphology of tissue repair* Int Rev Cytol 22:63-145

Mecham RP, Lange G, Madaras J, Stracher BC (1981) *Elastin synthesised by ligamentum nuchae fibroblasts: effects of culture conditions and extracellular matrix on elastin production* J Cell Biol 90:332-338

Mecham RP, Heuser JE (1991) *The elastic fibre* in "Cell Biology of the ECM" Ed. Hay Chp 3

Mecham RP, Davis EC (1994) In :Extracelular matrix assembly and structure Yurchenko PD, Birk DE, Mecham RP, eds. p281-314 San Diego :Academic press

Mecham RP (1999) In :Guidebook to the ECM, anchor and adhesion proteins Kreis J, Vale R, eds. p414-416 Oxford University Press

Mensing H, Czarnetski BM (1984) *Leukotriene B_4 induces in vitro fibroblast chemotaxis* J Invest Derm 82:9-12

Meredith JE Jr, Fazeli B, Scwhartz MA (1993) *The extracellular matrix as a cell survival factor* Mol Biol Cell 4:953-961

Messent AJ, Tuckwell DS, Knauper V, Humphries MJ, Murphy G, Gavrilovic J (1998) *Effects of collaganse-cleavage of type I collagen on $\alpha_2\beta_1$ integrin-mediated cell adhesion* J Cell Sci 111:1127-1135

Meyer-Ingold W, Eichner W (1995) *Platelet-derived growth factor* Cell Biol Inter 19:389-398

Mian M, Beghe F, Mian E (1992) *Collagen as a pharmacological approach in wound healing* Int J Tissue React XIV (S) 1-9

Michalopoulos GK, Defrances MC (1997) *Liver Regeneration* Science 276:60-66

Miller EJ, Gay S (1982) Collagen: an overview Meth Enzymol 82: 3-32

Miller EJ, Rhodes RK (1982) Preparation and characterisation of the different types of collagen Meth Enzymol 82: 33-64

Miller EJ, Rhodes RK, Furuto DK (1983) *Identification of collagen chains as a function of cyanogen bromide peptide patterns using gel permeation HPLC* Collagen Rel Res 3:79-87

Mimni ME (1988) *Collagen: biochemistry, biomechanics, biotechnology* Critical Reviews Chemistry

Minafra I, Luparello C, Sciarrino S, Tomasino SM, Minafra S (1985) *Quantitative determination of collagen types present in the ductal infiltrating carcinoma of human mammary gland* Cell Biol Int Rep 9:291-296

Minafra S, Giambelluca C, Andriob M, Pucci-Minafra I (1995) *Cell-cell and cell-collagen interactions influence gelatinase production by human breast-carcinoma cell line* Inter J Cancer 62:777-783

Mingatti P, Rifkin DB, Welgus HG, Parks WC (1996) In :The Molecular and Cellular Biology of wound repair Clark RAF, Henson PM, eds. p427-474 NY :Plenum Press

Miyake S, Yagita H, Maruyama T, Hashimoto H, Miyasaka N, Okumura K (1993) *Beta I integrin-mediated interaction with ECM proteins regulates cytokine gene expression in synovial fluid cells of rheumatoid arthritis patients* J Exp Med 177:863-868

Miyata T, Rubin AL, Stenzel KH, Dum MW (1979) *Collagen drug delivery service* USA Patent no. 4164559

Mizuno M, Kitafima T, Tomita M, Kuboki Y (1996) *The osteoblastic MC 3T3-E1 cells synthesised C-terminal propeptide of type I collagen, which promoted cell-attachment of osteoblasts* Biochem Biophys Acta 1310:97-102

Mohri H (1996) *Fibronectin and Integrins Interactions* J Invest Med 44:429-441

Morgan SJ, Darling DC eds. (1993) Animal Cell Culture BIOS Scientific Publishers: London

Morton LF, Fitzimmons CM, Rauterberg J, Barnes MJ (1987) *Platelet reactive sites in collagen. Collagen I and III possess different aggregatory sites* Biochem J 248:373-377

Morton LF, Zijenah LS, McCulloch C, Knight G, Humphries MJ, Barnes MJ (1991) *Integrin-dependent platelet recognition sites in collagen: Identification of a short-reactive sequence in the type III-derived fragment α1(III) CB3* Biochem Soc Trans 19:439S

Mosher DF (1993) *Assembly of fibronectin into extracellular matrix* Curr Opin Struc Biol 3:214-223

Muir H (1983) *Proteoglycans as organisers of the extracellular matrix* Biochem Soc Trans 11:613-622

Murphy CF, Orgill DP, Yannis IV (1990) *Partial dermal regeneration is induced by biodegradable collagen-glycosaminoglycan grafts* Lab Invest 63:305-315

Murphy G, Knauper V (1997) *Relating matrix metalloproteinase structure to function: why the "hemopexin" domain?* Matrix 15:511-518

Murphy G, Reynolds JJ (1993) *Extracellular matrix degradation* In :ECM and inheritable disorders of connective tissue Royce & Steinmann, eds. Chpt 9 NY: Wiley-Liss

Mutsaers SE, Bishop JE, McGrouther G, Laurent GJ (1997) *Mechanisms of tissue repair: from wound healing to fibrosis* Int J Biochem Cell Biol 29:5-17

Myers JC, Li D, Bageris A, Abraham V, Dion AS, Amenta PS (1997) *Biochemical and immunohistochemical characterisation of human type XIX defines a novel class of basement membrane zone collagens* Am J Pathol 151:1729-1740

Nagase H, Meng QI, Malinovski V, Huang W, Chung L, Bode W, Maskos K, Brew K (1999) *Engineering of selective TIMPs* Ann NY Acad Sci

Naito A, Tuzi S, Hazime S (1994) *A high resolution ^{15}N solid-state NMR study of collagen and related polypeptides. The effect of hydration on formation of interchain hydrogen bonds as the primary source of the collagen-type triple helix* Eur J Biochem 224:729-34

Nakamura M, Mishima H, Nishid T, Otori T (1994) *Binding of hyaluronan to plasma fibronectin increases the attachment of corneal epithelial cells to a fibronectin matrix* J Cell Phy 159:415-22

Nanney LB, King LE (1996) *Epidermal growth factor and transforming growth factor-α*: In :The Molecular and Cellular Biology of wound repair Clark RAF, Henson PM, eds. p171 –194 NY :Plennum Press

Needham DM (1952) *Adenosine triphosphate and structural proteins in relation to muscle contraction* Adv Enzymol 13:151-197

Negro A, Garbisa S, Gotte L, Spina M (1987) *The use of reverse-phase high performance liquid chromatography and precolumn derivatisation with dansyl chloride for quantitation of specific amino acids in collagen and elastin* Anal Biochem 160:39-46

Nobes CD, Hall A (1999) *Rho GTPases control polarity, protrusion and adhesion during cell movement* J Cell Biol 144:1235-1244

Noble PW, McKee CM, Horton MR (1998) *Induction of inflammatory gene expression by low-molecular weight hyaluronan fragments in macrophages* In: The chemistry, biology and medical applications of hyaluronan and its derivatives Ed. Laurent TC. London: Portland Press 219-225

Norris DA, Clark RA, Swigart LM, Huff JC, Weston WL, Howell SE (1982) *Fibronectin fragment(s) are chemotactic for human peripheral blood monocytes* J Immunol 129:1612-1618

O'Kane S, Ferguson MWJ (1997) *Transforming growth factor β's and wound healing* Int J Biochem Cell Biol 29:63-78

Olberg A, Antonsson P, Hedbom E, Heinegard D (1990) *Structure and function of extracellular matrix proteoglycans* Biochem Soc Trans 18:789-792

Oliver MH, Harrison NK, Bishop JE, Cole PJ, Laurent GJ (1986) *A rapid and convenient assay for counting cells cultured in microwell plates: application for assessment of growth factors* J Cell Sci 92:513-518

Olsen BR, ed. (1989) In :The Next Frontier: Molecular Biology of ECM NY:Dekker

Olsen BR, Ninomiya Y (1999) In :Guidebook to the ECM, anchor and adhesion proteins Kreis J, Vale R, eds. p380-408 Oxford University Press

Oxlund M, Manschot J, Viidik A (1988) *The role of elastin in the mechanical properties of the skin* J Biomech 21:213-218

Pachence JM, Berg RA, Silver FA (1986) *The role of collagen in the medical device industry* Med Dev Diag Indust 9:48-54

Pachence JM (1996) *Collagen-based devices for soft tissue repair* J Bio Mat Res 33:35-40

Partridge SM, Elsden DF, Thomas J (1963) *Constitution of the cross-linkages of elastin* Nature 197:1297-1298

Peacock EE, ed. (1984) In :Wound Repair p1-102. Saunders Company Ltd: Philadelphia

Peltonen J, Kaakkola S, Gay K, Olsen DR, Chu ML, Uitto J (1989) *Expression of extracellular matrix genes by cultured human cells: localisation of messenger RNAs and antigenic epitopes* Anal Biochem 178:184-193

Perris R, Syfrig J, Paulsson M, Bronner-Fraser M (1993) *Molecular mechanisms of neural crest cell attachment and migration of type I an IV collagen* J Cell Sci 106:1357-68

Pfeffer W (1884) Unters Bot Inst Tubingen 1:363-370

Pierce GF, Mustoe TA (1995) *Pharmacologic enhancement of wound healing* Ann Rev Med 46:467-481

Piez KA, Eibner EA, Lewis MS (1963) *The chromatographic separation of amino acid composition of the subunits of several collagens* Biochem 2:58-66

Piez KA (1997) *History of extracellular matrix: a personal view* Matrix Biol 16:85-92

Piez KA, Miller A (1974) *The structure of collagen fibrils* J Supramol Struct 2:121-137

Pihlajaniemi T, Rehn M (1995) *Two new collagen subgroups. Membrane-associated collagens and types XV and XVIII* Prog Nucleic Acid Res Mol Biol 50:225-262

Postlethwaite AE, Kang AH (1976) *Collagen and collagen peptide induced chemotaxis of human blood monocytes* J Exp Med 143:1299-1307

Postlethwaite AE, Seyer JM, Kang AH (1978) *Chemotactic attraction of human fibroblasts to type I, II and III collagens and collagen-derived peptides* Proc Nat Acad Sci 75:871-875

Postlethwaite AE, Snyderman R, Kang AH (1979) *Generation of a fibronectin chemotactic factor in serum by activation of complement* J Clin Invest 64:1379-1385

Postlethwaite AE, Keski-oja J, Balian G, Kang AH (1981) *Induction of fibroblast chemotaxis by fibronectin* J Exp Med 153:494-499

Postlethwaite AE, Kang AH (1988) *Fibroblast Chemoattractants* Meth Enzymol 163:697-707

Potts JR, Campbell ID (1994) *Fibronectin structure and assembly* Curr Opin Cell Biol 6:648-655

Powell DW, Mifflin RC, Valentich JD, Crowe SE, Saada JI, West AB (1999) *Myofibroblasts. I. Paracrine cells important in health and disease* Am J Physiol 277: C1-9

Probst CW, Bright RM (1985) *Wound Healing* In :Textbook of Small Animal Surgery Slatter DH, ed. vol 1:28-37 Saunders Company Ltd

Prockop DJ, Kivirikklo KJ (1995) *Collagens: Molecular biology, diseases and potentials for therapy* Ann Rev Biochem 64:403-434

Quinn JV (1996) *Clinical wound evaluation* Acad Emerg Med 3:298-299

Quinn JV, ed. (1998) In :Tissue Adhesives in wound care BC Decker Inc:London

Quintarelli G, Starcher BC, Vocaturo A, Di Gianfilippo F, Gotte L, Mecham RP (1979) *Fibrogenesis and biosynthesis of elastin in cartilage* Conn Tiss Res 7:1-19

Racine-Samson L, Rockey DC, Bissell DM (1997) *The role of the alpha 1 beta 1 integrin in wound contraction. A quantitative analysis of liver myofibroblasts in vivo and in primary culture* J Biol Chem 272:30911-30917

Raghow R (1994) *The role of the extracellular matrix and post inflammatory wound healing and fibrosis* FASEB J 8:823-31

Raif-Preminger M, Caplan SR, Yuli I (1995) *Temporal segregation in signalling: a novel mechanism in human neutrophils* Biochem Biophys Acta 1265:49-54

Ramachandran GN, Kartha G (1954) *Structure of collagen* Nature 174:269-270

Ramachandran GN, Kartha G (1955) *Structure of collagen* Nature 176:593-595

Ramachandran GN, Reddi AH, eds. (1976) In :Biochemistry of collagen NY:Plenum Press

Ramachandran GN (1988) *Stereochemistry of collagen* Int J Pept Prot Res 31:1-16

Ricard-Blum S, Dublet B, van der Rest M, eds. (2000) In: Unconventional collagens : Types VI, VII, VIII, IX, X, XII, XIV, XVI and XIX Oxford University Press

Rickard KA, Taylor J, Rennard SI, Spurzem JR (1993) *Migration of bovine bronchial epithelial cells to extracellular matrix components* Am J Resp Cell Mol Biol 8:63-68

Riley DJ, Kerr S, Guss SF, Curran SF, Laskin DL, Berg RA (1984) *Intratracheal instillation of collagen peptides induces a neutrophil influx in rats lungs* Trans Assoc Am Phyd XCVII 97:290-295

Robert L (1996) *Ageing of the vascular wall and atherogenesis: role of the elastin-laminin receptor* Atherosclerosis 123:169-179

Roberts AB, Sporn MB (1993) *Physiological actions and clinical applications of transforming growth factor-beta (TGF-β)* Growth Factors 8:1-9

Robertson TA, Maley MAL, Grounds MD, Papadimitriou JM (1993) *The role of macrophages in skeletal muscle regeneration with particular reference to chemotaxis* Exp Cell Res 267:321-331

Robson MC (1991) *Growth factors as wound healing agents* Curr Opin Biotech 2:863-867

Romberger DJ (1997) *Fibronectin* Int J Biochem Cell Biol 29:939-943

Rosenbloom J, Abrams WR, Mecham RP (1993) *Extracellular matrix 4: The elastic fibre* FASEB J 7:1208-1218

Rosenbloom J, Bashir M, Yeh H, Rosenbloom J, Ornstein-Goldstein N, Fazio M, Kahari V, Vitto J (1991) *Regulation of elastin gene expression* Ann NY Acad Sci 624:116-1136

Rossi A, Zuccarello LV, Zanaboni G, Monzani E, Dyne KM, Cetta G, Tenni R (1996) *Type I collagen CNBr peptides: species and behaviour in solution* Biochem 35:6048-6057

Ruhl M, Sahin E, Johannsen M, Somasundaram R, Manski D, Riecken EO, Schuppan D (1999) *Soluble collagen VI derives serum-starved fibroblasts through S phase and prevents apoptosis via down-regulation of Bax* J Biol Chem 274:34361-34368

Ruoslahti E, Pierschbacher MD (1987) *New perspectives in cell adhesion: RGD and integrins* Science 238:491-497

Ruoslahti E (1991) *Integrins as receptors for extracellular matrix* In :Cell biology of the ECM. Hay ED, Ed. Chp 10 p343 London :Plenum Press

Ruoslahti E, Noble NA, Kagami S, Border WA (1994) *Integrins* Kid Int 45:S44 S17-S22

Ruszczak Z, Schwartz RA (1999) *Collagen uses in dermatology – an update* Dermatology 199:285-289

Saelman EU, Horton LF, Barnes MJ, Graclnick HR, Hese KM, Nieuwenhuis HK, de Groot PG, Sixma JJ (1993) *Platelet adhesion to cyanogen-bromide fragments of collagen alpha 1(I) under flow conditions* Blood 82:3029-3033

Sakai LY (1995) *The Extracellular Matrix* Sci Med 2:58-67

Sakon M, Moden M, Gotoh M, Kobaipshi K, Kambayayashi J, Mori T, Okamura J (1989) *Use of microcrystalline collagen powder and fibrinogen tissue adhesive for haemostasis and prevention of rebleeding patients with hepatocellular carcinoma associated with cirrhosis of the liver* Surg Gynecol Obstet 168:453-454

Sandberg LB, Weissman N, Smith DW (1969) *The purification and partial characterisation of soluble elastin-like protein from copper-deficient porcine aorta* Biochem 8:2940-2945

Sandford KK (1948) *The growth in vitro of single isolated tissue cells* J Natl Cancer Institute 9:229-246

Santoro SA (1986) *Identification of a 160,000 dalton platelet membrane protein that mediates the initial divalent cation-dependent adhesion of platelets to collagen* Cell 46:913-920

Santoro SA, Walsh JJ, Staatz WD, Baranski KJ (1991) *Distinct determinants on collagen support $\alpha_2\beta_1$ integrin-mediated platelet adhesion and platelet activation* Cell Regul 2:905-913

Sasaki T, Timpl R (1999) In :Guidebook to the ECM, anchor and adhesion proteins Kreis J, Vale R, eds. p434-443 Oxford University Press

Schaffer M, Barbul A (1998) *Lymphocyte function in wound healing and following injury* B J Surg 85:444-60

Scheid A, Meuli M, Gassmann M, Wenger RH (2000) *Genetically modified mouse models in studies on cutaneous wound healing* Exp Physiol 85:687:704

Schiffman E, Gallin JI (1979) *Biochemistry of phagocyte chemotaxis* In: Current topics in cellular regulation Eds. Harker & Statmen Vol 15:203-261

Schwarzbauer JE, Patel RS, Fonda D, Hynes RO (1987) *Multiple sites of alternative splicing of the rat fibronectin gene transcript* EMBO J 6:2573-2580

Scott JE (1995) *ECM, supramolecular organisation and shape* J Anatomy 187:256-269

Senior RM, Griffin GL, Mecham RP (1982) *Chemotactic responses of fibroblasts to tropoelastin and elastin-derived peptides* J Clin Invest 70:614-618

Senior RM, Griffin GL, Mecham RP, Wrenn DS, Prasad KU, Urry DW (1984) *Val-Gly-Val-Ala-Pro-Gly, a repeating hexapeptide in elastin is chemotactic for fibroblasts and monocytes* J Cell Biol 99:870-874

Seppa HE, Yamada KM, Seppa ST, Silver MH, Kleinman HK, Schiffmann E (1981) *The cell binding fragment of fibronectin is chemotactic for fibroblasts* Cell Biol Int Rep 5:813-9

Seppa HE, Grotendorst G, Seppa S, Schiffmann E, Martin GR (1982) *Platelet-derived growth factor is chemotactic for fibroblasts* J Cell Biol 92:584-588

Seyer JM, Hutcheson ET, Kang AH (1972) *Collagen polymorphism in normal and cirrhotic human liver* J Clin Invest 59:241-248

Shah M, Revis D, Herrick S, Baillie R, Thorgeirson S, Ferguson M, Roberts A (1999) *Role of elevated plasma TGB-β levels in wound healing* Am J Pathol 154:1115-1124

Shahan Ta, Fawazi A, Bellon G, Monboisse JC, Kefalides NA (2000) *Regulation of tumor cell chemotaxis by Type IV collagen is mediated by a Ca^{2+} -dependent mechanism requiring CD47 and the integrin $\alpha_v\beta_3$* J Biol Chem 275:4796-4802

Shattil SJ, Kashiwagi H, Pampori N (1998) *Intergin Signalling:The platelet paradigm* Blood 91:2645-2657

Shingelton WD, Hodges DI, Brick P, Cawston TE (1996) *Collagenase: a key enzyme in collagen turnover* Biochem Cell Biol:74:759-775

Siegbaln A, Hammacher A, Westermark B, Heldin CH (1990) *Differential effects of the various isoforms of PDGF on chemotaxis of fibroblasts, monocytes and granulocytes* J Clin Invest 85:916-920

Silver A (1969) *The correlation between choline acetyltransferase and acetylcholinesterase activity in rat and pig* J Neurochem 16:386-96

Singer AJ, Clark RAF (1999) *Mechanisms of disease :cutaneous wound repair* N Eng J Med 341:738-746

Slavin J (1995) *Fibroblast growth factors: at the heart of angiogenesis* Cell Biol Inter 19:431-444

Slavin J (1996) *The role of cytokines in wound healing* J Pathol 178:5-10

Slavkin HC (2000) *The body's skin frontier and the challenges of wound healing: keloids* J Am Dental Assoc 131:362-365

Smith RS, Smith TJ, Blieden TM, Phipps RP (1997) *Fibroblasts as sentinel cells: synthesis of chemokines and regulation of inflammation* Am J Pathol 151:317-322

Soo C, Rahbar G, Moy R (1993) *The immunogenicity of bovine collagen implants* J Dermatol Surg Oncol 19:431-434

Staatz WD, Fok KF, Zutter MM, Adams SD, Rodriguez BA, Santoro SA (1991) *Identification of a tetrapeptide recognition sequence of the alpha 2 beta 1 integrin* J Biol Chem 266:7363-7367

Staatz WD, Rajpara SM, Wayner EA, Carter WG, Santoro SA (1989) *The membrane glycoprotein Ia-IIa (VLA-2) complex mediates the Mg^{2+} dependent adhesion of platelets to collagen* J Cell Biol 108:1917-1924

Staatz WD, Walsh JJ, Pexton JJ, Santoro SA (1990) *The $\alpha_1\beta_1$ integrin cell surface collagen receptor binds to the $\alpha 1(I)$-CB3 peptide of collagen* J Biol Chem 265:4778-4781

Stark M, Kuhn K (1968) *The properties of molecular fragments obtained on treating calf skin collagen with collagenase from Clostridium histolyticum* Eur J Biochem 6:534-541

Steenfos HH (1994) *Growth factors and wound healing* Scan J Plast Reconstr Hand Surg 28:95-108

Stenn KS, Madri JA, Tinghitella T, Terranova VP (1983) *Multiple mechanisms of dissociated epidermal cell spreading* J Cell Biol 96:63-67

Stenzil KH, Miyata T, Rubin AL (1974) *Collagen as a biomaterial* Ann Rev Biophys Bioeng 3:231-253

Sternlicht MD, Werb Z (1999) In :Guidebook to the ECM, anchor and adhesion proteins Kreis J, Vale R, eds. p505-538 Oxford University Press

Takashima A, Grinnell F (1985) *Fibronectin-mediated keratinocyte migration and initiation of fibronectin receptor function in vitro* J Invest Dermat 85:304-308

Tanzer ML, Chandrasekaran S, Dean JW, Giniger M (1993) *Role of laminin carbohydrates on cellular interactions* Kidney Inter 43:66-72

Terranova VP, Di Florio R, Hujanen ES, Lyall RM, Liott LA, Thorgeirsson U, Siegal GP, Schiffman E (1986) *Laminin promotes rabbit neutrophil motility and attachment* Am Soc Clin Invest 77:1180-1186

Terranova VP, Di Florio R, Hujanen ES, Lyall RM, Liott LA, Thorgeirsson U, Siegal GP, Schiffman E (1986) *Laminin promotes rabbit neutrophil motility and attachment* Am Soc Clin Invest 77:1180-1190

Thomas J, Elsden DF, Partridge SM (1963) *Degradation products from elastin. Partial structure of two major degradation products from the crosslinkages of elastin* Nature 200:651-652

Thoumine O, Nerem RM, Girard PR (1995) *Changes in organisation and composition of the extracellular matrix underlying cultured endothelial cells exposed to laminar steady shear stress* Lab Invest 73:565-576

Timpl R, Chu ML (1994) In :Extracellular Matrix Assembly and Structures. Yurchenco PD, Birk DE, Mecham RD, eds. San Diego :Academic p207-242

Todaro GJ, Green H (1963) *Quantitiative studies on the growth of mouse embryos in culture and their development into established lines* J Cell Biol 17:299-313

Toole BP (1997) *Hyaluronan in morphogenesis* J Intern Med 24:35-40

Traub W, Piez KA (1971) *The chemistry and structure of collagen* Adv Prot Chem 25:243-352

Trojanavska M, Le Roy EC, Eckes B, Trieg T (1997) *Pathogenesis of fibrosis: type I collagen and the skin* J Mol Med 76:266-74

Tryggvason K (1993) *The Laminin family* Curr Opin Cell Biol 5:877-882

Tryggvason K (1995) *Molecular properties and diseases of collagen* Kidney Inter V47 S49 S-24-28

Tuckwell DS, Reid KB, Barnes MJ, Humphries MJ (1996) *The A-domain of integrin α_2 binds specifically to a range of collagens but is not a general receptor for the collagens motif* Eur J Biochem 241:732-739

Tuhkanen AL, Tammi M, Pelttari A, Agren UM, Tammi R (1998) *Ultrastructural analysis of human epidermal CD44 reveals preferential distribution on plasma domains facing the hyaluronan-rich matrix pouches* J Histochem Cytochem 46:241-248

Tummalapalli CM, Tyagi SC (1999) *Responses of vascular smooth muscle cell to extracellular matrix degradation* J Cell Biochem 75:515-527

Turk JI, Heather CJ, Diengdoh JV (1966) *A histochemical analysis of mononuclear cell infiltrates of the skin with particular reference to delayed hypersensitivity in the guinea pig* Int Arch Allergy Appl Immunol 30:248-256

Uings IJ, Farrow SN (2000) *Cell receptors and cell signalling* Mol Pathol 53:295-299

van der Rest M, Bennett HPJ, Solomon S, Glorieux FH (1980) *Separation of collagen cyanogen bromide-derived peptides by reverse-phase* Biochem J 191:253-256

van der Rest M, Fietzek PP (1982) *A comprehensive approach to the study of collagen primary structure based on HPLC* Eur J Biochem 125:491-496

van der Rest M, Garrone R (1991) *Collagen family of proteins* FASEB J 5:2814-2823

Vandenberg P, Kern A, Ries A, Lickenbill-Edds L, Mann K, Kuhn K (1991) *Site with affinity to the $\alpha_1\beta_1$ and $\alpha_1\beta_2$ integrins* J Biol Chem 113:1475-1483

Vankemmelbeke M, Dekeyser PM, Hollander AP, Buttle DJ, Demeester J (1998) *Characterisation of helical cleavages in type II collagen generated by matrixins* Biochem J 330:633-640

Vogel BE, Tarone G, Giancotti FG, Gailit J, Ruoslahti E (1990) *A novel fibronectin receptor with an unexpected subunit composition* J Biol Chem 265:5935-5937

Vrhovski B, Weiss AS (1998) *Biochemistry of tropoelastin* Eur J Biochem 258:1-18

Wachi H, Seyama Y, Yamashita S, Suganami H, Uemura Y, Okamoto K, Yamada H, Tajima S (1995) *Stimulation of cell proliferation and autoregulation of elastin expression by elastin peptide VPGVG in cultures chick vascular smooth muscle cells* FEBS Lett 368:215-219

Wahl SM, Allen JB, Hines KL, Imamichi T, Wahl AM, Furcht LT, McCarthy JB (1994) *Synthetic fibronectin peptides suppress arthritis in rats by interrupting leukocyte adhesion and recruitment* J Clin Invest 94:655-662

Wall RT, Cooper SL, Kosek JC (1982) *The influence of exogenous fibronectin on blood granulocyte adherence to vascular endothelium in vitro* Cell Res 140:105-109

Watson S, Gibbins J (1998) *Collagen receptor signalling in platelets: extending the role of the ITAM* Immunol Today 19:260-264

Weber KT, Sun Y, Katawa LC, Cleutjens JPM (1995) *Connective Tissue: a metabolic entity* Mol Cell Cardiol 27:107-120

Weber KT, Sun Y, Laxmansa CK (1997) *Myofibroblasts and local angiotensin II in rat cardial tissue repair* Int J Biochem Cell Biol 29:31-42

Weiss JB, Ayad (1982) In :Collagen in health and disease Weiss JB, Jayson MIV, eds. Edinburgh :Churchill Livingstone

Welch MD, Odland GF, Clark RAF (1990) *Temporal relationships of F-actin bundle formation, collagen and fibronectin matrix assembly, and fibronectin receptor expression to wound contraction* J Cell Biol 110:133-145

Werb TN, Banda MJ, McKerrow JH, Sandhaus RA (1982) *Elastases and elastin degradation* J Inv Dermat 79:S1 154-159

West DC, Shaw DM, Lorenz P, Adzick NS, Longaker MT (1997) *Fibrotic healing of adult and late gestation fetal wounds correlates with increased hyaluronidase activity and removal of hyaluronan* Int J Biochem Cell Biol 29:201-210

Wight TN, Kinsella MG, Qwarnstrum EE (1992) *The role of proteoglycans in cell adhesion, migration and proliferation* Curr Opin Cell Biol 4:793-801

Wilkinson PC (1988) *Chemotaxis: An overview* Meth Enzymol 162:127-132

Wilkinson PC, Haston WS (1988) *Chemotaxis: An overview* Meth Enzymol 162:3-16

Wilkinson PC (1998) *Assay of leukocyte locomotion and chemotaxis* J Imm Methods 216:139-153

Willard H, Merritt L, Dean JA, Settle FA eds.(1988) Instrumental methods of analysis Wandsworth Publishing Co.

Winter P (1968) Animal models for biomedical research Nat Acad Sci: Washington

Woessner J (1991) *Matrix metalloproteinases and their inhibitors in connective tissue* FASEB J 5:2145-2154

Yamada KM, Hayashi, Akiyama SK (1982) *Structure and function of fibronectin* In :Extracellular matrix Hawkes & Wang, eds. p25-34 London :Plenum Press

Yamada KM (1991) *Fibronectin and other cell interactive glycoproteins* In: Cell Biology of the ECM Ed. Hay p111-146

Yamada KM (1997) *Integrin signalling* Matrix Biology 16:137-141

Yamada KM, Geiger B (1997) *Molecular interactions in cell adhesion complexes* Curr Opin Cell Biol 9:76-85

Yamamoto K, Yamamoto M, Noumura T (1992) *Disassembly of F-actin filaments in human endothelial cells cultured on type V collagen* Exp Cell Res 201:55-63

Yannas IV, Burke JF (1980) *Design of an artificial skin* J Biomed Mat Res 14:65-81

Yomemasu K, Nakanishi A, Sasaki T, Kashiba S (1983) *Stimulation of locomotion of peripheral blood monocytes by human plasma fibronectin* Microbiol Immunol 27:283-90

Young B, Heath JW, eds. (2000) In: Weater's functional histology: a text and colour atlas Churchill Livingston: Edinburgh

Ziaie Z, Fawzi A, Bellon G, Monboisse JC, Kefalides NA (1999) *A peptide of the α3 chain of Type IV collagen protects the basement membrane against damage to PMN* Biochem Biophys Res Comm 261:247-250

Zigmond SH, Hirsch JG (1973) *Leukocyte locomotion and chemotaxis. New methods for evaluation and demonstration of a cell-derived chemotactic factor* J Exp Med 137:387-410

Zijenah LS, Barnes MJ (1990) *Platelet-reactive sites in human collagens I and III: evidence for cell-recognition sites in collagen unrelated to RGD and like sequences* Thromb Res 59:553-566

Appendix

Publications:

Patent application filed 17/1/97

C. Hamilton & K.N. Broadley

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